# B12

Applicant: Parkin et al.
Serial No.: 09/766,344
Title: MEANS AND METHODS FOR MONITORING PROTEASE INHIBITOR ANTIRETROVIRAL THERAPY AND GUIDING THERAPEUTIC DECISIONS IN THE TREATMENT OF HIV/AIDS
Attorney Docket No.: 11068-033-999

CAJD: 363324.1

1/2

(54) Title: MEANS AND METHODS FOR MONITORING PROTEASE INHIBITOR ANTIRETROVIRAL THERAPY AND GUIDING THERAPEUTIC DECISIONS IN THE TREATMENT OF HIV/AIDS

(57) Abstract: This invention relates to antiviral drug susceptibility and resistance tests to be used in identifying effective drug regimens for the treatment of human immunodeficiency virus (HIV) infection and acquired immunodeficiency syndrome (AIDS), particularly treatment regimens including a protease inhibitor. The invention further relates to the means and methods of monitoring the clinical progression of HIV infection and its response to antiretroviral therapy using phenotypic or genotypic susceptibility assays.

## MEANS AND METHODS FOR MONITORING PROTEASE INHIBITOR ANTIRETROVIRAL THERAPY AND GUIDING THERAPEUTIC DECISIONS IN THE TREATMENT OF HIV/AIDS

This application claims the benefit of U.S. Application No. 09/874,472 filed June 4, 2001, and PCT International Application No. PCT/US02/01682, filed January 18, 2002, the contents of which are hereby incorporated by reference into this application.

5 Throughout this application, various references are referred to within parenthesis. Disclosures of these publications in their entireties are hereby incorporated by reference into this application to more fully describe the state of the art to which this invention pertains.

10

### Technical Field

This invention relates to antiretroviral drug susceptibility and resistance tests to be used in identifying effective drug regimens for the treatment of human immunodeficiency virus

15 (HIV) infection and acquired immunodeficiency syndrome (AIDS). The invention further relates to the means and methods of monitoring the clinical progression of HIV infection and its response to antiretroviral therapy using phenotypic or genotypic susceptibility assays. The invention also relates to

20 novel vectors, host cells and compositions for carrying out phenotypic susceptibility tests. The invention further relates to the use of various genotypic methodologies to identify patients who do not respond to a particular antiretroviral drug regimen. This invention also relates to the screening of

25 candidate antiretroviral drugs for their capacity to inhibit viral replication,

selected viral sequences and/or viral proteins. More particularly, this invention relates to the determination of protease inhibitor (PRI) susceptibility using phenotypic or genotypic susceptibility tests. This invention also relates to a means and method for accurately and reproducibly measuring viral replication fitness.

## Background of the Invention

HIV infection is characterized by high rates of viral turnover throughout the disease process, eventually leading to CD4 depletion and disease progression. Wei X, Ghosh SK, Taylor ME, et al. (1995) Nature 343, 117-122 and Ho DD, Naumann AU, Perelson AS, et al. (1995) Nature 373, 123-126. The aim of antiretroviral therapy is to achieve substantial and prolonged suppression of viral replication. Achieving sustained viral control is likely to involve the use of sequential therapies, generally each therapy comprising combinations of three or more antiretroviral drugs. Choice of initial and subsequent therapy should, therefore, be made on a rational basis, with knowledge of resistance and cross-resistance patterns being vital to guiding those decisions. The primary rationale of combination therapy relates to synergistic or additive activity to achieve greater inhibition of viral replication. The tolerability of drug regimens will remain critical, however, as therapy will need to be maintained over many years.

In an untreated patient, some $10^{10}$ new viral particles are produced per day. Coupled with the failure of HIV reverse transcriptase (RT) to correct transcription errors by exonucleolytic proofreading, this high level of viral

-3-

turnover results in $10^4$ to $10^5$ mutations per day at each position in the HIV genome. The result is the rapid establishment of extensive genotypic variation. While some template positions or base pair substitutions may be more error prone (Mansky LM, Temin HM (1995) J Virol 69, 5087-5094) (Schinazi RF, Lloyd RM, Ramanathan CS, et al. (1994) Antimicrob Agents Chemother 38, 268-274), mathematical modeling suggests that, at every possible single point, mutation may occur up to 10,000 times per day in infected individuals.

For antiretroviral drug resistance to occur, the target enzyme must be modified while preserving its function in the presence of the inhibitor. Point mutations leading to an amino acid substitution may result in changes in shape, size or charge of the active site, substrate binding site or in positions surrounding the active site of the enzyme. Mutants resistant to antiretroviral agents have been detected at low levels before the initiation of therapy. (Mohri H, Singh MK, Ching WTW, et al. (1993) Proc Natl Acad Sci USA 90, 25-29) (Nájera I, Richman DD, Olivares I, et al. (1994) AIDS Res Hum Retroviruses 10, 1479-1488) (Nájera I, Holguin A, Quiñones-Mateu E, et al. (1995) J Virol 69, 23-31). However, these mutant strains represent only a small proportion of the total viral load and may have a replication or competitive disadvantage compared with wild-type virus. (Coffin JM (1995) Science 267, 483-489). The selective pressure of antiretroviral therapy provides these drug-resistant mutants with a competitive advantage and thus they come to represent the dominant quasi species (Frost SDW, McLean AR (1994) AIDS 8, 323-332) (Kellam P, Boucher CAB, Tijnagal JMGH (1994) J

Gen Virol 75, 341-351) ultimately leading to a rebound in viral load in the patient.

Early development of antiretroviral therapy focused on inhibitors of reverse transcriptase. Both nucleoside and non-nucleoside inhibitors of this enzyme showed significant antiviral activity (DeClerq, E. (1992) AIDS Res. Hum. Retrovir. 8:119-134). However, the clinical benefit of these drugs had been limited due to drug resistance, limited potency, and host cellular factors (Richman, D.D. (1993) Ann. Rev. Pharm. Tox. 32:149-164). Thus inhibitors targeted against a second essential enzyme of HIV were urgently needed.

In 1988, the protease enzyme of HIV was crystallized and its three-dimensional structure was determined, (Navia MA, Fitzgerald PMD, McKeever BM, Leu CT, Heimbach JC, Herber WK, Sigal IS, Darke PL, Springer JP (1989) Nature 337:615-620 and Winters MA, Schapiro JM, Lawrence J, Merigan TC (1997) In Abstracts of the International Workshop on HIV Drug Resistance, Treatment Strategies and Eradication, St. Petersburg, Fla.) allowing for the rapid development of protease inhibitors. Initially, it was hypothesized that HIV protease, unlike reverse transcriptase, would be unable to accommodate mutations leading to drug resistance. This is not the case, and to date over 20 amino acid substitutions in the HIV protease have been observed during treatment with the currently available protease inhibitors. The genetic pattern of mutations conferring resistance to these protease inhibitors is complex, and cross-resistance between structurally different compounds occurs.

-5-

## PROTEASE INHIBITORS

HIV protease was classified as an aspartic proteinase on the basis of putative active-site homology (Toh H, Ono M, Saigo K, Miyata T (1985) Nature 315:691), its inhibition by peptastin (Richards AD, Roberts R, Dunn BM, Graves MC, Kay J (1989) FEBS Lett 247:113), and its crystal structure (Navia MA, Fitzgerald PMD, McKeever BM, Lau CT, Heimbach JC, Herber WK, Sigal IS, Darke PL, Springer JP (1989) Nature 337:615-620). The enzyme functions as a homodimer composed of two identical 99-amino acid chains (Debouck C, Navia MA, Fitzgerald PMD, McKeever BM, Leu CT, Heimbach JC, Herber WK, Sigal IS, Darke PL, Springer JP (1988) Proc. Natl. Acad. Sci. USA 84:8903-8906), with each chain containing the characteristic Asp-Thr-Gly active-site sequence at positions 25 to 27 (Toh H, Ono M, Saigo K, Miyata T (1985) Nature 315:691).

HIV protease processes gag (p55) and gag-pol (p160) polyprotein products into functional core proteins and viral enzymes (Kohl NE, Diehl RE, Rands E, Davis LJ, Hanobik MG, Wolanski B, Dixon RA (1991) J. Virol. 65:3007-3014 and Kramer RA, Schaber MD, Skalka AM, Ganguly K, Wong-Staal F, Reddy EP (1986) Science 231:1580-1584). During or immediately after budding, the polyproteins are cleaved by the enzyme at nine different cleavage sites to yield the structural proteins (p17, p24, p7, and p6) as well as the viral enzymes reverse transcriptase, integrase, and protease (Pettit SC, Michael SF, Swanstrom R (1993) Drug Discov. Des. 1:69-83).

An asparagine replacement for aspartic acid at active-site residue 25 results in the production of noninfectious

-6-

viral particles with immature, defective cores (Huff JR
(1991) AIDS J. Med. Chem. 34:2305-2314, Kaplan AH, Zack
JA, Knigge M, Paul DA, Kempf DJ, Norbeck DW, Swanstrom R
(1993) J. Virol. 67:4050-4055, Kohl NE, Emini EA, Schleif
WA, Davis LJ, Heimbach JC, Dixon RA, Scolnik EM, Sigal IS
(1988) Proc. Natl. Acad. Sci. USA 85:4686-4690, Peng C, Ho
BK, Chang TW, Chang NT (1989) J. Virol. 63:2550-2556).
Similarly, wild-type virus particles produced by infected
cells treated with protease inhibitors contain unprocessed
precursors and are noninfectious (Crawford S, Goff SP
(1985) J. Virol. 53:899-907, Gottlinger HG, Sodroski JG,
Haseltine WA (1989) Proc. Natl. Acad. Sci. USA
86:5781-5785, Katoh IY, Yoshinaka Y, Rein A, Shibuya M,
Odaka T, Oroszlan S (1985) Virology 145:280-292, Kohl NE,
Emini EA, Schleif WA, Davis LJ, Heimbach JC, Dixon RA,
Scolnik EM, Sigal IS (1988) Proc. Natl. Acad. Sci. USA
85:4686-4690, Peng C, Ho BK, Chang TW, Chang NT (1989) J.
Virol. 63:2550-2556, Stewart L, Schatz G, Wogt VM (1990)
J. Virol. 64:5076-5092). Unlike reverse transcriptase
inhibitors, protease inhibitors block the production of
infectious virus from chronically infected cells (Lambert
DM, Petteway, Jr. SR, McDanal CE, Hart TK, Leary JJ,
Dreyer GB, Meek TD, Bugelski PJ, Bolognesi DP, Metcalf BW,
Matthews TJ (1992) Antibicrob. Agents Chemother.
36:982-988). Although the viral protease is a symmetric
dimer, it binds its natural substrates or inhibitors
asymmetrically (Dreyer, GB, Boehm JC, Chenera B,
DesJarlais RL, Hassell AM, Meek TD, Tomaszek TAJ, Lewis M
(1993) Biochemistry 32:937-947, Miller MJ, Schneider J,
Sathyanarayana BK, Toth MV, Marshall GR, Clawson L, Selk
L, Kent SB, Wlodawer A (1989) Science 246:1149-1152).
These findings together with the knowledge that amide

bonds of proline residues are not susceptible to cleavage by mammalian endopeptidases gave rise to the first class of HIV-1 protease inhibitors based on the transition state mimetic concept, with the phenylalanine-proline cleavage

5    site being the critical nonscissile bond .(Roberts NA, Martin JA, Kinchington D, Broadhurst AV, Craig JC, Duncan IB, Galpin SA, Handa BK, Kay J, Krohn A, Lambert RW, Merett JH, Mills JS, Parkes KEB, Redshaw S, Ritchie AJ, Taylor DL, Thomas GJ, Machin PJ (1990) Science

10   248:358-361).

**Amino acids implicated in resistance to protease inhibitors.**

15   As new protease inhibitors are developed, the ability of certain amino acid substitutions to confer resistance to the inhibitor is usually determined by several methods, including selection of resistant strains in vitro, site-directed mutagenesis, and determination of amino acid

20   changes that are selected during early phase clinical trials in infected patients. While some amino acid substitutions are specifically correlated with resistance to certain protease inhibitors (see below), there is considerable overlap between sets of mutations implicated

25   in resistance to all approved protease inhibitors. Many investigators have attempted to classify these mutations as either being "primary" or "secondary", with varying definitions. For example, some investigators classify as primary mutations which are predicted, based on X-ray

30   crystallographic data, to be in the enzyme active site with the potential for direct contact with the inhibitor (e.g. D30N, G48V, I50V, V82A/F/S/T, I84V, N88S, L90M).

Secondary mutations are usually considered as being compensatory for defects in enzyme activity imposed by primary mutations, or as having enhancing effects on the magnitude of resistance imparted by the primary mutations (e.g. L10I/F/R/V, K20I/M/R/T, L24I, V32I, L33F/V, M36I/L/V, M46I/L/V, I47V, I54L/V, L63X, A71T/V, G73A/S/T, V77I, N88D). Lists of mutations and *corresponding* inhibitors are maintained by several organizations, for example: Schinazi et al., Mutations in retroviral genes associated with drug resistance, *Intl. Antiviral News* 1999,7:46-69 and Shafer et al., Human Immunodeficiency Virus Reverse Transcriptase and Protease Sequence Database, Nucleic Acids Research 1999, 27(1), 348-352 (also accessible via the internet at http://www.viral-resistance.com/ or http://hivdb.stanford. edu/hiv/)

## Saquinavir

Saquinavir, developed by Hoffmann-La Roche, was the first protease inhibitor to undergo clinical evaluation, demonstrating that HIV-1 protease was a valid target for the treatment of HIV infection (Jacobsen H, Brun-Vezinet F, Duncan I, Hanggi M, Ott M, Vella S, Weber J, Mous J (1994) J. Virol. 68:2016-2020). Saquinavir is a highly active peptidomimetic protease inhibitor with a 90% inhibitory concentration (IC90) of 6 nM (id). In vitro, saquinavir can select for variants with one or both of two amino acid substitutions in the HIV-1 protease gene, a valine-for-glycine substitution at position 48 (G48V), a methionine-for-leucine substitution at residue 90 (L90M), and the double substitution G48V-L90M (Eberle J, Bechowsky B, Rose D, Hauser U, vonder Helm K, Guertler L, Nitschko H (1995) AIDS Res. Hum. Retroviruses 11:671-676, Jacobsen H, Yasargil K, Winslow DL, Craig JC, Kroehn A, Duncan IB, Mous J (1995) Virology 206:527-534, Turriziani O, Antonelli G, Jacobsen H, Mous J, Riva E, Pistello M, Dianzani F (1994) Acta Virol. 38:297-298). In most cases, G48V is the first mutation to appear, and continued selection results in highly resistant double-mutant variants. A substitution at either residue results in a 3- to 10-fold decreased susceptibility to the inhibitor, whereas the simultaneous occurrence of both substitutions causes a more severe loss of susceptibility of >100-fold (id).

In vivo, saquinavir therapy appears to select almost exclusively for mutations at codons 90 and 48 (id, Jacobsen H, Hangi M, Ott M, Duncan IB, Owen S, Andreoni M, Vella S, Mous J (1996) J. Infect. Dis. 173:1379-1387, Vella S, Galluzzo C, Giannini G, Pirillo MF, Duncan I,

Jacobsen H, Andreoni M, Sarmati L, Ercoli L (1996) Antiviral Res. 29:91-93). Saquinavir-resistant variants emerge in approximately 45% of patients after 1 year of monotherapy with 1,800 mg daily (Craig IC, Duncan IB,
5     Roberts NA, Whittaker L (1993) In Abstracts of the 9th International Conference on AIDS, Berlin, Germany, Duncan IB, Jacobsen H, Owen S, Roberts NA (1996) In Abstracts of the 3rd Conference of Retroviruses and Opportunistic Infections, Washington, D.D., id, Mous J, Brun-Vezinet F,
10    Duncan IB, Haenggi M, Jacobsen H, Vella S (1994) In Abstracts of the 10th International Conference on AIDS, Yokohama, Japan). The frequency of resistance is lower (22%) in patients receiving combination therapy with zidovudine, zalcitabine, and saquinavir (Collier AC,
15    Coombs R, Schoenfeld DA, Bassett RL, Joseph Timpone MS, Baruch A, Jones M, Facey K, Whitacre C, McAuliffe VJ, Friedman HM, Merigan TC, Reichmann RC, Hooper C, Corey L (1996) N. Engl. J. Med. 334:1011-1017). In contrast to in vitro-selected virus, where the G48V mutation is the first
20    step to resistance, the L90M exchange is the predominant mutation selected in vivo while the G48V (2%) or the double mutant (<2%) is rarely found (id). In another recent study of in vivo resistance during saquinavir monotherapy no patient was found to harbor a G48V mutant
25    virus (Ives KJ, Jacobsen H, Galpin SA, Garaev MM, Dorrell L, Mous J, Bragman K, Weber JN (1997 J. Antimicrob. Chemother. 39:771-779). Interestingly, Winters et al. (id) observed a higher frequency of the G48V mutation in patients receiving higher saquinavir doses as monotherapy.
30    All patients (six of six) who initially developed G48V also acquired a V82A mutation either during saquinavir treatment or after switching to either indinavir or

nelfinavir. An identical mutational pattern was found in another study during saquinavir monotherapy (Eastman PS, Duncan IB, Gee C, Race E (1997) In Abstracts of the International Workshop on HIV Drug Resistance, Treatment Strategies and Eradication, St. Petersburg, Fla.). Some residues represent sites of natural polymorphism of the HIV-1 protease (positions 10, 36, 63, and 71) and appear to be correlated to the L90M mutation (id). Another substitution, G73S, has been recently identified and may play a role in saquinavir resistance in vivo. Isolates from five patients with early saquinavir resistance and those from two patients with induced saquinavir resistance after a switch of therapy to indinavir carried the G73S and the L90M substitutions Dulioust A, Paulous S, Guillemot L, Boue F, Galanaud P, Clavel F (1997) In Abstracts of the International Workshop on HIV Drug Resistance, Treatment Strategies and Eradication, St. Petersburg, Fla.).

**Ritonavir**

Ritonavir, developed by Abbott Laboratories, was the second HIV protease inhibitor to be licensed in the United States. Ritonavir is a potent and selective inhibitor of HIV protease that is derived from a C2-symmetric, peptidomimetic inhibitor (Ho DD, Toyoshima T, Mo H, Kempf DJ, Norbeck D, Chen CM, Wideburg NE, Burt SK, Erickson JW, Singh MK (1994) J. Virol. 68:2016-2020). In vitro activity has been demonstrated against a variety of laboratory strains and clinical isolates of HIV-1 with IC90s of 70 to 200 nM (Kuroda MJ, El-Farrash MA, Cloudhury S, Harada S (1995) Virology 210:212-216.

-12-

Resistant virus generated by serial in vitro passages is associated with specific mutations at positions 84, 82, 71, 63, and 46 (Markowitz M, Mo H, Kempf DJ, Norbeck DW, Bhat TN, Erickson JW, Ho DD (1995) J. Virol. 69:701-706).

5   The I84V substitution appeared to be the major determinant of resistance, resulting in a 10-fold reduction in sensitivity to ritonavir.   Addition of the V82F mutation confers an even greater level of resistance, up to 20-fold.   The substitutions M46I, L63P, and A71V, when

10   introduced into the protease coding region of wild-type NL4-3, did not result in significant changes in drug susceptibility.   Based   on   replication   kinetics experiments, these changes are likely to be compensatory for   active-site   mutations,   restoring   the   impaired

15   replicative   capacity   of   the   combined   V82F   and   I84V mutations.

## Indinavir

Indinavir, developed by Merck & Co., is the third HIV protease inhibitor licensed in the United States. Indinavir is a potent and selective inhibitor of HIV-1 and HIV-2 proteases with Ki values of 0.34 and 3.3 nM, respectively (Vacca Jp, Dorsey BD, Schleif WA, Levin RB, McDaniel SL, Darke PL, Zugay J, Quintero JC, Blahy OM, Roth E, Sardana VV, Schlabach AJ, Graham PI, Condra JH, Gotlib L, Holloway MK, Lin J, Chen L-w, Vastag K, Ostobich D, Anderson PS, Emini EA, Huff JR (1994) Proc. Natl. Acad. Sci. USA 91:4096-4100). The drug acts as peptidomimetic transition state analogue and belongs to the class of protease inhibitors known as HAPA (hydroxyaminopentane amide) compounds (ibid). Indinavir provides enhanced aqueous solubility and oral bioavailability and in cell culture exhibits an IC95 of 50 to 100 nM (Emini EA, Schleif WA, Deutsch P, Condra JH (1996) Antiviral Chemother. 4:327-331.

Despite early reports of a lack of in vitro resistance by selection with indinavir (id), Tisdale et al. (Tisdale M, Myers RE, Maschera B, Parry NR, Oliver NM, Blair ED (1995) Antibicrob. Agents Chemother. 39:1704-1710) were able to obtain resistant variants during selection in MT-4 cells with substitutions at residues 32, 46, 71, and 82. At least four mutations were required to produce a significant loss of susceptibility (6.1-fold compared with the wild type). The mutation at position 71, described as compensatory (Markowitz M, Mo H, Kempf DJ, Norbeck DW, Bhat TN, Erickson JW, Ho DD (1995) J. Virol. (id), appeared to contribute phenotypic resistance and also to improve virus growth. Emini et al. (id) and Condra et al.

(Condra JH, Holder DJ, Schleif WA, Blahy OM, Danovich RM,
Gabryelski LJ, Graham DJ, Laird D, Quintero JC, Rhodes A,
Robbins HL, Roth E, Shivaprakash M, Yang T, Chodakewitz
JA, Deutsch PJ, Leavitt RY, Massari Fe, Mellors JW,
Squires KE, Steigbigel RT, Teppler H, Emini EA (1995)
Nature 374:569-571) found by constructing mutant HIV-1
clones that at least three mutations at residues 46, 63,
and 82 were required for the phenotypic manifestation of
resistance with a fourfold loss of susceptibility.

-15-

## Nelfinavir

Nelfinavir, developed by Agouron Pharmaceuticals, is a selective, nonpeptidic HIV-1 protease inhibitor that was designed by protein structure-based techniques using

5   iterative protein crystallographic analysis (Appelt KR, Bacquet J, Bartlett C, Booth CLJ, Freer ST, Fuhry MM, Gehring MR, Herrmann SM, Howland EF, Janson CA, Jones TR, Kan CC, Kathardekar V, Lewis KK, Marzoni GP, Mathews DA, Mohr C, Moomaw EW, Morse CA, Oatley SJ, Ogden RC, Reddy

10   MR, Reich SH, Schoettlin WS, Smith WW, Varney MD, Villafranca JE, Ward RW, Webber S, Webber SE, Welsh KM, White J (1991) J. Med. Chem. 34:1925-1928). In vitro, nelfinavir was found to be a potent inhibitor of HIV-1 protease with a Ki of 2.0 nM (Kaldor SW, Kalish VJ, Davies

15   JF, Shetty BV, Fritz JE, Appelt K, Burgess JA, Campanale KM, Chirgadze NY, Clawson DK, Dressman BA, Hatch SD, Khalil DA, Kosa MB, Lubbehusen PP, Muesing MA, Patrick AK, Reich SH, Su KS, Tatlock JH (1997) J. Med. Chem. 40:3979-3985). The drug demonstrated antiviral activity

20   against several laboratory and clinical HIV-1 and HIV-2 strains with 50% effective concentrations ranging from 9 to 60 nM (Patick AK, Boritzki TJ, Bloom LA (1997) Antimicrob. Agents Chemother. 41:2159-2164). Nelfinavir exhibits additive-to-synergistic effects when combined

25   with other antiretroviral drugs (Partaledis JA, Yamaguchi AK, Tisdale M, Blair EE, Falcione C, Maschera B, Myers RE, Pazhanisamy S, Futer O, Bullinan AB, Stuver CM, Byrn RA, Livingston DJ (1995) J. Virol. 69:5228-5235). Preclinical data showed high levels of the drug in mesenteric lymph

30   , nodes and the spleen and good oral bioavailability (Shetty BV, Kosa MB, Khalil DA, Webber S (1996) Antimicrob. Agents Chemother. 40:110-114).

In *vitro*, following 22 serial passages of HIV-1$_{NL4-3}$ in the presence of nelfinavir, a variant (P22) with a sevenfold reduced susceptibility was isolated. After an additional six passages a variant (P28) with a 30-fold-decreased susceptibility to nelfinavir was identified (Patick AK, Ho H, Markowitz M, Appelt K, Wu B, Musick L, Kaldor S, Reich S, Ho D, Webber S (1996) Antimicrob. Agents Chemother. 40:292-297). Sequence analysis of the protease gene from these variants identified in decreasing frequency the substitutions D30N, A71V, and I84V for the P22 variant and mutations M46I, I84V/A, L63P, and A71V for the P28 variant. Antiviral susceptibility testing of recombinant mutant HIV-1$_{NL4-3}$ containing various mutations resulted in a fivefold-increased 90% effective concentration for the I84V and D30N single mutants and the M46I/I84V double mutant, whereas no change in susceptibility was observed with M46I, L63P, or A71V alone (*ibid*).

**Amprenavir**

Amprenavir is a novel protease inhibitor developed by Vertex Laboratories and designed from knowledge of the HIV-1 protease crystal structure (Kim EE, Baker CT, Dyer MD, Murcko MA, Rao BG, Tung RD, Navia MA (1995) J. Am. Chem. Soc. 117:1181-1182). The drug belongs to the class of sulfonamide protease inhibitors and has been shown to be a potent inhibitor of HIV-1 and HIV-2, with IC50s of 80 and 340 nM, respectively. The mean IC50 for amprenavir against clinical viral isolates was 12 nM (St. Clair MH, Millard J, Rooney J, Tisdale M, Parry N, Sadler BM, Blum MR, Painter G (1996) Antiviral Res. 29:53-56). HIV-1 variants 100-fold resistant to amprenavir have been selected by in vitro passage experiments (id). DNA sequence analysis of the protease of these variants revealed a sequential accumulation of point mutations resulting in amino acid substitutions L10F, M46I, I47V, and I50V. The key resistance mutation in the HIV-1 protease substrate binding site is I50V. As a single mutation it confers a two- to threefold decrease in susceptibility (ibid). The other substitutions did not result in reduced susceptibility when introduced as single mutations into an HIV-1 infectious clone (HXB2). However, a triple protease mutant clone containing the mutations M46I, I47V, and I50V was 20-fold less susceptible to amprenavir than wild-type virus. The I50V mutation has not been frequently reported in resistance studies with other HIV protease inhibitors. Kinetic characterization of these substitutions demonstrated an 80-fold reduction in the inhibition constant ($K_i$) for the I50V single-mutant protease and a 270-fold-reduced $K_i$ for the triple mutant M46I/I47V/I50V, compared to the wild-type enzyme

-18-

(Pazhanisamy S, St6uvr CM, Cullinan AB, Margolin N, Rao BG (1996) J. Biol. Chem. 271:17979-17985). The single mutants L10F, M46I, and I47V did not display reduced affinity for amprenavir. The catalytic efficiency $(k_{cat}/K_m)$ of the I50V mutant was decreased up to 25-fold, while the triple mutant M46I/I47V/I50V had a 2-fold-higher processing efficiency than the I50V single mutant, confirming the compensatory role of the M46I-and-I47V mutation. The reduced catalytic efficiency $(k_{cat}/K_m)$ for these mutants in processing peptides appeared to be due to both increased $K_m$ and decreased $k_{cat}$ values.

## VIRAL FITNESS

The relative ability of a given virus or virus mutant to replicate is termed viral fitness. Fitness is dependent on both viral and host factors, including the genetic composition of the virus, the host immune response, and selective pressures such as the presence of anti-viral compounds. Many drug-resistant variants of HIV-1 are less fit than the wild-type, i.e. they grow more slowly in the absence of drug selection. However, since the replication of the wild-type virus is inhibited in the presence of drug, the resistant mutant can outgrow it. The reduction in fitness may be a result of several factors including: decreased ability of the mutated enzyme (i.e. PR or RT) to recognize its natural substrates, decreased stability of the mutant protein, or decreased kinetics of enzymatic catalysis. See Back et al., EMBO J. 15: 4040-4049, 1996; Goudsmit et al., J. Virol. 70: 5662-5664, 2996; Maschera et al., J. Biol. Chem. 271: 33231-33235, 1996; Croteau et al., J. Virol. 71: 1089-1096, 1997; Zennou et al., J. Virol. 72: 300-3306, 1998; Harrigan et al., J. Virol. 72:

3773-3778, 1998; Kosalaraksa et al., J. Virol. 73: 5356-5363, 1999; Gerondelis et al., J. Virol. 73: 5803-5813, 1999. Drug resistant viruses that are less fit than wild type may be less virulent i.e. they may cause damage to the host immune system more slowly than a wild type virus. Immunological decline may be delayed after the emergence of drug resistant mutants, compared to the rate of immunological decline in an untreated patient. The defect causing reductions in fitness may be partially or completely compensated for by the selection of viruses with additional amino acid substitutions in the same protein that bears the drug resistance mutations (for example, see Martinez-Picado et al., J. Virol. 73:3744-3752, 1999), or in other proteins which interact with the mutated enzyme. Thus, amino acids surrounding the protease cleavage site in the gag protein may be altered so that the site is better recognized by a drug-resistant protease enzyme (Doyon et al., J. Virol. 70: 3763-3769, 1996; Zhang et al., J. Virol. 71: 6662-6670, 1997; Mammano et al., J, Virol. 72: 7632-7637, 1998).

## INTEGRASE

Integration of viral DNA into the host chromosome is a necessary process in the HIV replication cycle (Brown, P.O., 1997, in *Retroviruses;* Coffin, J.M., Hughes, S.H. & Varmus, H.E., eds., Cold Spring Harbor Lab. Press, Plainview, NY, 161-203). The key steps of DNA integration are carried out by the viral integrase protein, which, along with protease and reverse transcriptase, is one of three enzymes encoded by HIV. Combination antiviral therapy with protease and reverse transcriptase inhibitors has demonstrated the potential therapeutic efficacy of

antiviral therapy for treatment for AIDS (Vandamme, A.M.,
Van Vaerenbergh, K. & De Clerq, E., 1998, *Antiviral Chem.
Chemother.* 9, 187-203). However, the ability of HIV to
rapidly evolve drug resistance, together with toxicity
problems, requires the development of additional classes
of antiviral drugs. Integrase is an attractive target for
antivirals because it is essential for HIV replication
and, unlike protease and reverse transcriptase, there are
no known counterparts in the host cell. Furthermore,
integrase uses a single active site to accommodate two
different configurations of DNA substrates, which may
constrain the ability of HIV to develop drug resistance to
integrase inhibitors. However, unlike protease and
reverse transcriptase, for which several classes of
inhibitors have been developed and cocrystal structures
have been determined, progress with the development of
integrase inhibitors has been slow. A major obstacle has
been the absence of good lead compounds that can serve as
the starting point for structure-based inhibitor
development. Although numerous compounds have been
reported to inhibit integrase activity *in vitro*, most of
these compounds exhibit little specificity for integrase
and are not useful as lead compounds (Pommier, Y., Pilon,
A.A., Bajaj K, K., Mazumder, A. & Neamati, N., 1997,
*Antiviral Chem. Chemother* 8).

HIV-1 integrase is a 32-kDa enzyme that carries out DNA
integration in a two-step reaction (Brown, P.O., *ibid.*).
In the first step, called 3' processing, two nucleotides
are removed from each 3' end of the viral DNA made by
reverse transcription. In the next step, called DNA
strand transfer, a pair of transesterification reactions

integrates the ends of the viral DNA into the host genome. Integrase is comprised of three structurally and functionally distinct domains, and all three domains are required for each step of the integration reaction (Engelman, A. Bushman, F.D. & Craigie, R., 1993, *EMBO J.* 12, 3269-3275). The isolated domains form homodimers in solution, and the three-dimensional structures of all three separate dimers have been determined (Dyda, F., Hickman, A.B. Jenkins, T.M., Engelman, A., Craigie, R. & Davies, D.R., 1994, *Science* 226, 1981-1986; Goldgur, Y. Dyda, Hickman, A.B., Jenkins, T.M., Craigie, R. & Davies, D.R., 1998, *Proc. Natl. Acad. Sci., USA* 95, 9150-9154; Maignan, S., Guilloteau, J.P., Zhou-Liu, Q., Clement-Mella, C. & Mikol, V., 1998, *J Mol. Biol.* 282, 259-368; Lodi, P.J., Ernst, J.A., Kuszewski, J., Hickman, A.B., Engelman, A., Craigie, R., Clore, G.M. & Gronenborn, A.M. 1995 *Biochemistry* 34, 9826-9833; Eijkelenboom, A.P., Lutzke, R.A., Boelens, R., Plasterk, R.H., Kaptein, R. & Hard, K. 1995 *Nat. Struct. Biol.* 2, 807-810; Cai, M.L., Zheng, R., Caffrey, M., Craigie, R., Clore, G.M. & Gronenborn, A.M., 1997 *Nat. Struct. Biol.* 4, 839-840). Although little is known concerning the organization of these domains in the active complex with DNA substrates, integrase is likely to function as at least a tetramer (Dyda, F., Hickman, A.B. Jenkins, T.M., Engelman, A., Craigie, R. & Davies, D.R., 1994, *Science* 226, 1981-1986). Extensive mutagenesis studies mapped the catalytic site to the core domain (residues 50-212), which contains the catalytic residues D64, D116, and E152 (Engelman, A. & Craigie R., 1992, *J. Virol.* 66, 6361-6369; Kulkosky, J., Jones, K.S., Katz, R.A., Mack, J.P. & Skalka, A.M., 1992, *Mol. Cell Biol* 12, 2331-2338). The structure of this

domain of HIV-1 integrase has been determined in several crystal forms (Dyda, F., Hickman, A.B. Jenkins, T.M., Engelman, A., Craigie, R. & Davies, D.R., 1994, *Science* 226, 1981-1986; Goldgur, Y. Dyda, Hickman, A.B., Jenkins, T.M., Craigie, R. & Davies, D.R., 1998, *Proc. Natl. Acad. Sci.*, USA 95, 9150-9154; Maignan, S., Guilloteau, J.P., Zhou-Liu, Q., Clement-Mella, C. & Mikol, V., 1998, *J Mol. Biol.* 282, 259-368).

Hazuda et al. (Science 287: 646-650, 2000) have described compounds (termed L-731, 988 and L-708,906) which specifically inhibit the strand-transfer activity of HIV-1 integrase and HIV-1 replication in vitro. Viruses grown in the presence of these inhibitors display reduced inhibitor susceptibility and bear mutations in the integrase coding region at amino acid positions 66 (T66I), 153 (S153Y), and 154 (M154I). Site-directed mutants of a laboratory strain of HIV-1 (HXB2) with these amino acid changes confirmed their direct role in conferring reduced integrase inhibitor susceptibility. In addition some of these mutants displayed delayed growth kinetics, suggesting that viral fitness was impaired.

It is an object of this invention to provide a drug susceptibility and resistance test capable of showing whether a viral population in a patient is either more or less susceptible to a given prescribed drug. Another object of this invention is to provide a test that will enable the physician to substitute one or more drugs in a therapeutic regimen for viruses that show altered susceptibility to a given drug or drugs after a course of therapy. Yet another object of this invention is to

provide a test that will enable selection of an effective drug regimen for the treatment of HIV infections and/or AIDS. Yet another object of this invention is to provide the means for identifying alterations in the drug susceptibility profile of a patient's virus, in particular identifying changes in susceptibility to protease inhibitors. Still another object of this invention is to provide a test and methods for evaluating the biological effectiveness of candidate drug compounds which act on specific viruses, viral genes and/or viral proteins particularly with respect to alterations in viral drug susceptibility associated with protease inhibitors. It is also an object of this invention to provide the means and compositions for evaluating HIV antiretroviral drug resistance and susceptibility.

It is an object of this invention to provide a method for measuring replication fitness which can be adapted to viruses, including, but not limited to human immunodeficiency virus (HIV), hepadnaviruses (human hepatitis B virus), flaviviruses (human hepatitis C virus) and herpesviruses (human cytomegalovirus). This and other objects of this invention will be apparent from the specification as a whole.

-24-

## Summary of the Invention

The present invention relates to methods of monitoring, via phenotypic and genotypic methods the clinical progression of human immunodeficiency virus infection and its response to antiviral therapy. The invention is also based, in part, on the discovery that genetic changes in HIV protease (PR) which confer changes in susceptibility to antiretroviral therapy may be rapidly determined directly from patient plasma HIV RNA using phenotypic or genotypic methods. The methods utilize nucleic acid amplification based assays, such as polymerase chain reaction (PCR). Herein—after, such nucleic acid amplification based assays will be referred to as PCR based assays. This invention is based in part on the discovery of mutations at codons 10, 20, 36, 46, 63, 77 and 88 of HIV protease in PRI treated patients in which the presence of certain combinations of these mutations correlate with changes in certain PRI susceptibilities. This invention is also based on the discovery that susceptibility to HIV protease antivirals may not be altered even if primary mutations are present. Additional mutations at secondary positions in HIV protease are required for a reduction in virus susceptibility. This invention established for the first time that a mutation at position 82 of protease (V82A, F, S, or T) in the absence of another primary mutation was not correlated with a reduction in drug susceptibility. Decreased susceptibility to protease inhibitors, such as indinavir and saquinavir, in viruses containing V82A, F, S or T was observed in viruses with additional mutations at secondary positions, such as, 24, 71, 54, 46, 10 and/or 63 as described herein. Decreased susceptibility to protease

inhibitors, such as indinavir and saquinavir, in viruses containing V82A, F, S or T was also observed in viruses with at least 3 or more additional mutations at secondary positions. This inventions also established for the first time that a mutation at position 90 of protease (L90M) in the absence of another primary mutation was not correlated with a reduction in drug susceptibility. Decreased susceptibility to protease inhibitors, such as indinavir and saquinavir, in viruses containing L90M was observed in viruses with additional mutations at secondary positions, such as, 73, 71, 77, and/or 10 as described herein. Decreased susceptibility to protease inhibitors, such as indinavir and saquinavir, in viruses containing L90M was also observed in viruses with at least 3 or more additional mutations at secondary positions. The mutations were found in plasma HIV nucleic acid after a period of time following the initiation of therapy. The development of these mutations, or combinations of these mutations, in HIV PR was found to be an indicator of the development of alterations in phenotypic susceptibility/resistance, which can be associated with virologic failure and subsequent immunological response.

In one embodiment of the invention, a method of assessing the effectiveness of protease antiretroviral therapy of an HIV-infected patient is provided comprising:(a)collecting a plasma sample from the HIV-infected patient; (b) evaluating whether the plasma sample contains nucleic acid encoding HIV protease having a mutation at primary and secondary positions; and (c) determining changes in susceptibility to a protease inhibitor.

In a further embodiment of the invention, PCR based assays, including phenotypic and genotypic assays, may be used to detect a substitution at codon 88 from asparagine to a serine residue either alone or in combination with

5    one or more mutations at other codons selected from the group consisting of 10, 20, 36, 46, 63 and/or 77 or a combination thereof of HIV PR. A mutation at codon 88 from an asparagine residue to a serine residue (N88S) alone correlates with an increase in susceptibility to

10   amprenavir and a mutation at codon 88 from an asparagine residue to a serine residue in combination with mutations at codons 63 and/or 77 or a combination thereof correlates with an increase in susceptibility to amprenavir and a decrease in nelfinavir and indinavir susceptibility.

15

In a further embodiment of the invention, PCR based assays, including phenotypic and genotypic assays, may be used to detect mutations at codons 10, 20, 36, 46, 63, 77, and 88 of HIV PR which correlate with changes in

20   susceptibility to antiretroviral therapy and immunologic response. Once mutations at these loci have been detected in a patient undergoing PRI antiretroviral therapy, an alteration in the therapeutic regimen should be considered. The timing at which a modification of the

25   therapeutic regimen should be made, following the assessment of antiretroviral therapy using PCR based assays, may depend on several factors including the patient's viral load, CD4 count, and prior treatment history.

30

In a further embodiment of the invention, PCR based assays, including phenotypic and genotypic assays, may be

used to detect a substitution at codon 82 from valine to an alanine (V82A), phenylalanine (V82F), serine (V82S), or threonine (V82T) residue either alone or in combination with one or more mutations at other codons, referred to herein as secondary mutations, selected from the group consisting of 20, 24, 36, 71, 54, 46, 63 and/or 10 or a combination thereof of HIV PR. A mutation at codon 82 from a valine residue to a alanine, phenylalanine, serine or threonine alone correlates with susceptibility to certain protease inhibitors including indinavir and saquinavir. A mutation at codon 82 from a valine residue to a alanine, phenylalanine, serine or threonine in combination with secondary mutations at codons 24 and/or 71 or 20 and/or 36 correlates with a reduction in susceptibility to indinavir and saquinavir, respectively. A mutation at codon 82 from a valine residue to a alanine, phenylalanine, serine or threonine in combination with at least 3 secondary mutations correlates with a reduction in susceptibility to indinavir and saquinavir.

In a further embodiment of the invention, PCR based assays, including phenotypic and genotypic assays, may be used to detect a substitution at codon 90 from leucine to a methionine (L90M) residue either alone or in combination with one or more mutations at other codons, referred to herein as secondary mutations, selected from the group consisting of 73, 71, 46 and/or 10 or a combination thereof of HIV PR. A mutation at codon 90 from a leucine residue to a methionine alone correlates with susceptibility to certain protease inhibitors including indinavir and saquinavir. A mutation at codon 90 from a leucine residue to a methionine in combination with

secondary mutations at codons 73 and/or 71 or 73, 71 and/or 77 correlates with a reduction in susceptibility to indinavir and saquinavir, respectively. A mutation at codon 90 from a leucine residue to a methionine in

5   combination with at least 3 secondary mutations correlates with a reduction in susceptibility to indinavir and saquinavir.

In another aspect of the invention there is provided a method for assessing the effectiveness of a protease

10  inhibitor antiretroviral drug comprising: (a) introducing a resistance test vector comprising a patient-derived segment and an indicator gene into a host cell; (b) culturing the host cell from step (a); (c) measuring expression of the indicator gene in a target host cell

15  wherein expression of the indicator gene is dependent upon the patient derived segment; and (d) comparing the expression of the indicator gene from step (c) with the expression of the indicator gene measured when steps (a) - (c) are carried out in the absence of the PRI anti-HIV

20  drug, wherein a test concentration of the PRI, anti-HIV drug is presented at steps (a) - (c); at steps (b) - (c); or at step (c).

This invention also provides a method for assessing the

25  effectiveness of protease inhibitor antiretroviral therapy in a patient comprising: (a) developing a standard curve of drug susceptibility for an PRI anti-HIV drug; (b) determining PRI anti-HIV drug susceptibility in the patient using the susceptibility test described above; and

30  (c) comparing the PRI anti-HIV drug susceptibility in step (b) with the standard curve determined in step (a), wherein a decrease in PRI anti-HIV susceptibility

indicates development of anti-HIV drug resistance in the patient's virus and an increase in PRI anti-HIV susceptibility indicates drug hypersensitivity in the patient's virus.

This invention also provides a method for evaluating the biological effectiveness of a candidate PRI HIV antiretroviral drug compound comprising: (a) introducing a resistance test vector comprising a patient-derived segment and an indicator gene into a host cell; (b) culturing the host cell from step (a); (c) measuring expression of the indicator gene in a target host cell wherein expression of the indicator gene is dependent upon the patient derived segment; and (d) comparing the expression of the indicator gene from step (c) with the expression of the indicator gene measured when steps (a) - (c) are carried out in the absence of the candidate PRI anti-viral drug compound, wherein a test concentration of the candidate PRI anti-viral drug compound is present at steps (a) - (c); at steps (b) - (c); or at step (c).

The expression of the indicator gene in the resistance test vector in the target cell is ultimately dependent upon the action of the HIV enzymes (PR and RT) encoded by the patient-derived segment DNA sequences. The indicator gene may be functional or non-functional.

In another aspect this invention is directed to antiretroviral drug susceptibility and resistance tests for HIV/AIDS. Particular resistance test vectors of the invention for use in the HIV/AIDS antiretroviral drug susceptibility and resistance test are identified.

Yet another aspect of this invention provides for the identification and assessment of the biological effectiveness of potential therapeutic antiretroviral compounds for the treatment of HIV and/or AIDS. In another aspect, the invention is directed to a novel resistance test vector comprising a patient-derived segment further comprising one or more mutations on the PR gene and an indicator gene.

Still another aspect of this invention provides for the identification and assessment of the fitness of a virus infecting a patient. In another aspect, the invention is directed to a novel resistance test vector comprising a patient-derived segment further comprising one or more mutations on the PR gene and an indicator gene, enabling the measurement of viral fitness.

## Brief Description of the Drawings

Fig. 1

Resistance Test Vector. A diagrammatic representation of
the resistance test vector comprising a patient derived
segment and an indicator gene.


Fig. 2

Two Cell Assay. Schematic Representation of the Assay. A
resistance test vector is generated by cloning the
patient-derived segment into an indicator gene viral
vector. The resistance test vector is then co-transfected
with an expression vector that produces amphotropic murine
leukemia virus (MLV) envelope protein or other viral or
cellular proteins which enable infection. Pseudotyped
viral particles are produced containing the protease (PR)
and the reverse transcriptase (RT) gene products encoded
by the patient-derived DNA sequences. The particles are
then harvested and used to infect fresh cells. Using
defective PR and RT sequences it was shown that luciferase
activity is dependent on functional PR and RT. PR
inhibitors are added to the cells following transfection
and are thus present during particle maturation. RT
inhibitors, on the other hand, are added to the cells at
the time of or prior to viral particle infection. The
assay is performed in the absence of drug and in the
presence of drug over a wide range of concentrations.
Luciferase activity is determined and the percentage (%)
inhibition is calculated at the different drug
concentrations tested.

Fig. 3

Examples of phenotypic drug susceptibility profiles. Data
are analyzed by plotting the percent inhibition of
luciferase activity vs. log10 concentration. This plot is
5    used to calculate the drug concentration that is required
to inhibit virus replication by 50% (IC50) or by 95%
(IC95). Shifts in the inhibition curves towards higher
drug concentrations are interpreted as evidence of drug
resistance. Three typical curves for a nucleoside reverse
10   transcriptase inhibitor (AZT), a non-nucleoside reverse
transcriptase inhibitor (efavirenz), and a protease
inhibitor (indinavir) are shown. A reduction in drug
susceptibility (resistance) is reflected in a shift in the
drug susceptibility curve toward higher drug
15   concentrations (to the right) as compared to a baseline
(pre-treatment) sample or a drug susceptible virus
reference control, such as pNL4-3 or HXB-2, when a
baseline sample is not available.


20   Fig. 4

Phenotypic PRI susceptibility profile: patient 0732. A
PCR-based phenotypic susceptibility assay was carried out
giving the phenotypic drug susceptibility profile showing
decreased susceptibility to nelfinavir and indinavir, and
25   increased susceptibility to amprenavir.


Fig. 5

Phenotypic PRI susceptibility profile of a protease mutant
generated by site-specific oligonucleotide-directed
30   mutagenesis. A PCR-based phenotypic susceptibility assay
was carried out giving the phenotypic drug susceptibility
profile of a virus having substitutions at codons 63, 77

and 88 (L63P, V77I and N88S). The profile demonstrates resistance to both nelfinavir and indinavir, and increased susceptibility to amprenavir.

5    Figure 6. Distribution of saquinavir hyper-susceptibility by amino acid change at position 82.

Figure 7. Relative luciferase activity of integrase inhibitor-resistant site-directed mutants.

10

Fig. A

Two Cell Fitness Assay. Schematic Representation of the Fitness Assay. A fitness test vector is generated by cloning the patient-derived segment into an indicator gene

15   viral vector. The fitness test vector is then co-transfected with an expression vector that produces amphotropic murine leukemia virus (MLV) envelope protein or other viral or cellular proteins which enable infection. Pseudotyped viral particles are produced

20   containing the protease (PR) and the reverse transcriptase (RT) gene products encoded by the patient-derived DNA sequences. The particles are then harvested and used to infect fresh cells. Using defective PR and RT sequences it was shown that luciferase activity is dependent on

25   functional PR and RT. The fitness assay is typically performed in the absence of drug. If desired, the assay can also be performed at defined drug concentrations. Luciferase activity produced by patient derived viruses is compared to the luciferase activity produced by well-

30   characterized reference viruses. Replication fitness is expressed as a percent of the reference.

Figure B.

Determining the replication fitness of patient viruses. Virus stocks produced from fitness test vectors derived from patient samples were used to infect cells.

5   Luciferase activity was measured at various times after infection.  Patient derived viruses may produce more, approximately the same, or less luciferase activity than the reference virus (Ref) and are said to have greater,  equivalent,  or reduced replication  fitness,

10   respectively.  The drug susceptibility profiles of three representative patient derived viruses are shown (P1, P2, P3).

Figure C.

15   Identifying  alterations  in  protease  or  reverse transcriptase function associated with differences in replication fitness of patient viruses.   Replication fitness is expressed as a percent of the reference virus (top).    Fitness measurements are compared to protease

20   processing of the p55 gag polyprotein (middle) and reverse transcriptase activity (bottom). Protease processing is measured by Western blot analysis using an antibody that reacts with the mature capsid protein (p24).    The detection of unprocessed p55 or incompletely processed p41

25   polyproteins are indicators of reduced cleavage.   Reverse transcriptase activity is measured using a quantitative RT-PCR assay and is expressed as a percent of the reference virus.

30   Figure D.

Correlating reduced replication fitness with reduced reverse transcriptase activity.    Viruses containing

various amino acid substitutions at position 190 (A, S, C, Q, E, T, V) of reverse transcriptase were constructed using site directed mutagenesis. The reference virus contains G at this position. Replication fitness and

5

reverse transcriptase activities were compared.

Figure E.
Correlating reduced replication fitness with reduced
10    protease processing of p55 gag. Viruses containing various amino acid substitutions in protease (D30N, L90M, etc) were constructed using site directed mutagenesis. Replication fitness and p55 gag processing were compared.

15    Figure F.
Correlating reduced replication fitness with reduced drug susceptibility. A large collection (n=134) of patient samples were evaluated for phenotypic drug susceptibility and replication fitness. Replication fitness and drug
20    susceptibility were compared.

Figure G.
Relationship between protease inhibitor susceptibility and replication fitness. Patient samples were sorted based on
25    their replication fitness (<25% of reference, 26-75% of reference, and >75% of reference). Mean values for protease inhibitor susceptibility were determined for each fitness group and plotted for each drug and all drugs combined.

30

Figure H.
Relationship    between    reverse    transcriptase    inhibitor

susceptibility and replication fitness. Patient samples were sorted based on their replication fitness (<25% of reference, 26-75% of reference, and >75% of reference). Mean values for reverse transcriptase susceptibility were determined for each fitness group and plotted for each drug and all drugs combined.

Figure I.

Reduced replication fitness is associated with high numbers of protease mutations, and the L90M mutation. Patient viruses were sorted based on the number of protease mutations. Viruses with large numbers of protease mutations or the L90M protease mutation generally exhibit reduced replication fitness.

Figure J.

Low replication capacity is associated with specific protease mutations. Patient viruses were sorted based on replication capacity. Specific protease mutations either alone (D30N) or in combination (L90M plus others) were observed with high frequency in viruses with reduced replication fitness.

Figure K.

Relationship between nelfinavir susceptibility, protease processing and replication fitness. Patient viruses were sorted based on nelfinavir susceptibility (<10 or >10 of reference). Protease processing and replication fitness were plotted for all patient viruses. Viruses with reduced nelfinavir susceptibility generally exhibited reduced protease processing and reduced replication fitness.

Figure L.    Protease mutations associated with reduced protease processing.  Patient viruses were sorted based on protease processing.   Specific protease mutations were observed  at  high  frequency  in  viruses  with  reduced protease processing.


Figure M.

Representative patient sample exhibiting reversion to drug susceptibility  during  a  period  of  drug  treatment interruption.   Virus samples were collected weekly during a  period  of  treatment  interruption  and  evaluated  for phenotypic  drug  susceptibility.   Values  shown  represent fold  change  in  susceptibility  compared  to  the  reference virus.


Figure N.

Representative  patient  sample  exhibiting  increased replication  fitness  during  a  period  of  drug  treatment interruption.   Virus samples were collected weekly during a  period  of  treatment  interruption  and  evaluated  for phenotypic  drug  susceptibility.   Fitness  values  shown represent percent of the reference virus.  The increase in fitness between week 9 and week 10 corresponds to improved protease  processing  (bottom)  and  reversion  of  the  drug resistant  phenotype  to  a  drug  sensitive  phenotype  (Figure M).


Figure O.

Increased  replication  fitness  during  treatment interruption.   Replication fitness was measured at the time  of  treatment  interruption  and  various  times  during

-38-

the period of treatment interruption. Generally, replication fitness was significantly higher in samples that corresponded to timepoints after the virus had reverted from a drug resistant phenotype to a drug sensitive phenotype.

## Detailed Description of the Invention

The present invention relates to methods of monitoring the clinical progression of HIV infection in patients receiving antiretroviral therapy, particularly protease inhibitor antiretroviral therapy.

In one embodiment, the present invention provides for a method of evaluating the effectiveness of antiretroviral therapy of a patient comprising (i) collecting a biological sample from an HIV-infected patient; and (ii) determining whether the biological sample comprises nucleic acid encoding HIV PR having a mutation at one or more positions in the PR. The mutation(s) correlate positively with alterations in phenotypic susceptibility.

In a specific embodiment, the invention provides for a method of evaluating the effectiveness of PRI antiretroviral therapy of a patient comprising (i) collecting a biological sample from an HIV-infected patient; and (ii) determining whether the biological sample comprises nucleic acid encoding HIV PR having a mutation at codon 88 from an asparagine residue to a serine residue (N88S). This invention established, using a phenotypic susceptibility assay, that a mutation at

codon 88 to a serine residue of HIV protease is correlated with an increase in amprenavir susceptibility.

In a specific embodiment, the invention provides for a method of evaluating the effectiveness of PRI antiretroviral therapy of a patient comprising (i) collecting a biological sample from an HIV-infected patient; and (ii) determining whether the biological sample comprises nucleic acid encoding HIV PR having a mutation at codon 88 from an asparagine residue to a serine residue (N88S) either alone or in combination with mutations at codons 63 and/or 77 or a combination thereof. This invention established, using a phenotypic susceptibility assay, that a mutation at codon 88 to a serine residue of HIV protease is correlated with an increase in amprenavir susceptibility and a mutation at codon 88 to a serine residue in combination with mutations at codons 63 and/or 77 or a combination thereof of HIV protease are correlated with an increase in amprenavir susceptibility and a decrease in nelfinavir and indinavir susceptibility.

In a specific embodiment, the invention provides for a method of evaluating the effectiveness of PRI antiretroviral therapy of a patient comprising (i) collecting a biological sample from an HIV-infected patient; and (ii) determining whether the biological sample comprises nucleic acid encoding HIV PR having a mutation at codon 88 from an asparagine residue to a serine residue (N88S) either alone or in combination with mutations at codons 46, 63 and/or 77 or a combination thereof. This invention established, using a phenotypic

susceptibility assay, that a mutation at codon 88 to a
serine residue of HIV protease is correlated with an
increase in amprenavir susceptibility and a mutation at
codon 88 to a serine residue in combination with mutations
5    at codons 46, 63 and/or 77 or a combination thereof of HIV
protease are correlated with an increase in amprenavir
susceptibility and a decrease in nelfinavir and indinavir
susceptibility.


10   In a specific embodiment, the invention provides for a
method   of   evaluating   the   effectiveness   of   PRI
antiretroviral  therapy  of  a  patient  comprising  (i)
collecting  a  biological  sample  from  an  HIV-infected
patient;  and  (ii)  determining  whether  the  biological
15   sample  comprises  nucleic  acid  encoding  HIV  PR  having  a
mutation  at  codon  88  from  an  asparagine  residue  to  a
serine  residue  (N88S)  either  alone  or  in  combination  with
mutations  at  codons  10,  20,  36,  46,  63  and/or  77  or  a
combination thereof. This invention established, using a
20   phenotypic  susceptibility  assay,  that  a  mutation  at  codon
88  to  a  serine  residue  of  HIV  protease  is  correlated  with
an  increase  in  amprenavir  susceptibility  and  a  mutation  at
codon  88  to  a  serine  residue  in  combination  with  mutations
at  codons  10,  20,  36,  46,  63  and/or  77  or  a  combination
25   thereof  of  HIV  protease  are  correlated  with  an  increase  in
amprenavir  susceptibility  and  a  decrease  in  nelfinavir  and
indinavir susceptibility.


Under   the   foregoing   circumstances,   the   phenotypic
30   susceptibility  profile  and  genotypic  profile  of  the  HIV
virus  infecting  the  patient  has  been  altered  reflecting  a
change  in  response  to  the  antiretroviral  agent.  In  the

case of PRI antiretroviral therapy, the HIV virus infecting the patient may be resistant to one or more PRIs but hypersensitive to another of the PRIs as described herein. It therefore may be desirable after detecting the mutation(s), to either increase the dosage of the antiretroviral agent, change to another antiretroviral agent, or add one or more additional antiretroviral agents to the patient's therapeutic regimen. For example, if the patient was being treated with nelfinavir when the N88S mutation arose, the patient's therapeutic regimen may desirably be altered by either (i) changing to a different PRI antiretroviral agent, such as saquinavir, ritonavir or amprenavir and stopping nelfinavir treatment; or (ii) increasing the dosage of nelfinavir; or (iii) adding another antiretroviral agent to the patient's therapeutic regimen. The effectiveness of the modification in therapy may be further evaluated by monitoring viral burden such as by HIV RNA copy number. A decrease in HIV RNA copy number correlates positively with the effectiveness of a treatment regimen.

The phrase "correlates positively," as used herein, indicates that a particular result renders a particular conclusion more likely than other conclusions.

When reference is made to a particular codon number, it is understood that the codon number refers to the position of the amino acid that the codon codes for. Therefore a codon referencing a particular number is equivalent to a "postion" referencing a particular number, such as for example, "codon 88" or "position 88".

● ●

Another preferred, non-limiting, specific embodiment of the invention is as follows: A method of evaluating the effectiveness of PRI therapy of a patient comprising (i) collecting a biological sample from an HIV-infected patient; (ii) purifying and converting the viral RNA to cDNA and amplifying HIV sequences using HIV primers that result in a PCR product that comprises the PR gene; (iii) performing PCR using primers that result in PCR products comprising wild type or serine at codon 88; and (iv) determining, via the products of PCR, the presence or absence of a serine residue at codon 88.

Another preferred, non-limiting, specific embodiment of the invention is as follows: A method of evaluating the effectiveness of PRI therapy of a patient comprising (i) collecting a biological sample from an HIV-infected patient; (ii) purifying and converting the viral RNA to cDNA and amplifying HIV sequences using HIV primers that result in a PCR product that comprises the PR gene; (iii) performing PCR using primers that result in PCR products comprising wild type or serine at codon 88 and mutations at codons 63 and/or 77; and (iv) determining, via the products of PCR, the presence or absence of a serine residue at codon 88 and the presence or absence of mutations at codons 63 and/or 77.

Another preferred, non-limiting, specific embodiment of the invention is as follows: A method of evaluating the effectiveness of PRI therapy of a patient comprising (i) collecting a biological sample from an HIV-infected patient; (ii) purifying and converting the viral RNA to cDNA and amplifying HIV sequences using HIV primers that

result in a PCR product that comprises the PR gene; (iii) performing PCR using primers that result in PCR products comprising wild type or serine at codon 88 and mutations at codons 63, 77 and/or 46 or a combination thereof; and (iv) determining, via the products of PCR, the presence or absence of a serine residue at codon 88 and the presence or absence of mutations at codons 63, 77 and/or 46 or a combination thereof.

Another preferred, non-limiting, specific embodiment of the invention is as follows: A method of evaluating the effectiveness of PRI therapy of a patient comprising (i) collecting a biological sample from an HIV-infected patient; (ii) purifying and converting the viral RNA to cDNA and amplifying HIV sequences using HIV primers that result in a PCR product that comprises the PR gene; (iii) performing PCR using primers that result in PCR products comprising wild type or serine at codon 88 and mutations at codons 63, 77, 46, 10, 20, and/or 36 or a combination thereof; and (iv) determining, via the products of PCR, the presence or absence of a serine residue at codon 88 and the presence or absence of mutations at codons 63, 77, 46, 10, 20, and/or 36 or a combination thereof.

The presence of the mutation at codon 88 to a serine of HIV PR indicates that the effectiveness of the current or prospective PRI therapy may require alteration, since as shown by this invention mutation at codon 88 to a serine residue increases the susceptibility to amprenavir. Using the methods of this invention, changes in the PRI therapy would be indicated.

-44-

The presence of the mutation at codon 88 to a serine of alone or in combination with mutations at condons 63, 77, 46, 10, 20, and/or 36 or a combination thereof of HIV PR indicates that the effectiveness of the current or prospective PRI therapy may require alteration, since as shown by this invention a mutation at codon 88 to a serine residue alone increases the susceptibility to amprenavir and a mutation at codon 88 to a serine residue in combination with mutations at condons 63, 77, 46, 10, 20, and/or 36 or a combination increases the susceptibility to amprenavir but also reduces the susceptibility to nelfinavir and indinavir. Using the methods of this invention, changes in the PRI therapy would be indicated.

Another preferred, non-limiting, specific embodiment of the invention is as follows: a method of evaluating the effectiveness of antiretroviral therapy of an HIV-infected patient comprising: (a) collecting a biological sample from an HIV-infected patient; and (b) determining whether the biological sample comprises nucleic acid encoding HIV protease having a mutation at codon 88 to serine. Using the phenotypic susceptibility assay, it was observed that the presence of the mutation at codon 88 to serine of HIV PR causes a an increase in amprenavir susceptibility.

Another preferred, non-limiting, specific embodiment of the invention is as follows: a method of evaluating the effectiveness of antiretroviral therapy of an HIV-infected patient comprising: (a) collecting a biological sample from an HIV-infected patient; and (b) determining whether the biological sample comprises nucleic acid encoding HIV protease having a mutation at codon 88 to serine and

● ●

additional mutation(s) at codons 63 and/or 77 or a combination thereof. Using the phenotypic susceptibility assay, it was observed that the presence of the mutation at codon 88 to serine of HIV PR causes an increase in

5 amprenavir susceptibility and the presence of the mutations at codon 88 to serine in combination with a mutation at codon(s) 63 and/or 77 or a combination thereof of HIV PR causes a decrease in nelfinavir and indinavir susceptibility while increasing amprenavir susceptibility.

10

Another preferred, non-limiting, specific embodiment of the invention is as follows: a method of evaluating the effectiveness of antiretroviral therapy of an HIV-infected patient comprising: (a) collecting a biological sample

15 from an HIV-infected patient; and (b) determining whether the biological sample comprises nucleic acid encoding HIV protease having a mutation at codon 88 to serine and additional mutation(s) at codons 63, 77 and/or 46 or a combination thereof. Using the phenotypic susceptibility

20 assay, it was observed that the presence of the mutation at codon 88 to serine of HIV PR causes an increase in amprenavir susceptibility and the presence of the mutations at codon 88 to serine in combination with a mutation at codon(s) 46, 63 and/or 77 or a combination

25 thereof of HIV PR causes a decrease in nelfinavir and indinavir susceptibility while increasing amprenavir susceptibility.

Another preferred, non-limiting, specific embodiment of

30 the invention is as follows: a method of evaluating the effectiveness of antiretroviral therapy of an HIV-infected patient comprising: (a) collecting a biological sample

from an HIV-infected patient; and (b) determining whether the biological sample comprises nucleic acid encoding HIV protease having a mutation at codon 88 to serine and additional mutation(s) at codons 63, 77, 46, 10, 20 and/or

5   36 or a combination thereof. Using the phenotypic susceptibility assay, it was observed that the presence of the mutation at codon 88 to serine of HIV PR causes an increase in amprenavir susceptibility and the presence of the mutations at codon 88 to serine in combination with a

10  mutation at codon(s) 63, 77, 46, 10, 20 and/or 36 or a combination thereof of HIV PR causes a decrease in nelfinavir and indinavir susceptibility while increasing amprenavir susceptibility.

15  This invention also provides the means and methods to use the resistance test vector comprising an HIV gene and further comprising a PR mutation for drug screening. More particularly, the invention describes the resistance test vector comprising the HIV protease having a mutation at

20  codon 88 to a serine alone or in combination with mutations at codons 10, 20, 36, 46, 63 and/or 77 or a combination thereof for drug screening. The invention further relates to novel vectors, host cells and compositions for isolation and identification of the HIV-1

25  protease inhibitor resistant mutant and using such vectors, host cells and compositions to carry out anti-viral drug screening. This invention also relates to the screening of candidate drugs for their capacity to inhibit said mutant.

30

This invention provides a method for identifying a compound which is capable of affecting the function of the

protease of HIV-1 comprising contacting the compound with the polypeptide(s) comprising all or part of the HIV-1 protease, wherein codon 88 is changed to a serine residue, wherein a positive binding indicates that the compound is capable of affecting the function of said protease.

This invention also provides a method for assessing the viral fitness of patient's virus comprising: (a) determining the luciferase activity in the absence of drug for the reference control using the susceptibility test described above; (b) determining the luciferase activity in the absence of drug for the patient virus sample using the susceptibility test described above; and (c) comparing the luciferase activity determined in step (b) with the luciferase activity determined in step (a), wherein a decrease in luciferase activity indicates a reduction in viral fitness of the patient's virus.

If a resistance test vector is constructed using a patient derived segment from a patient virus which is unfit, and the fitness defect is due to genetic alterations in the patient derived segment, then the virus produced from cells transfected with the resistance test vector will produce luciferase more slowly. This defect will be manifested as reduced luciferase activity (in the absence of drug) compared to the drug sensitive reference control, and may be expressed as a percentage of the control.

In a further embodiment of the invention, PCR based assays, including phenotypic and genotypic assays, may be used to detect mutations at positions 20 and 88 of HIV PR, which correlate with a reduction in viral fitness and

immunological response.

It is a further embodiment of this invention to provide a means and method for measuring replication fitness for viruses, including, but not limited to human immunodeficiency virus (HIV), hepadnaviruses (human hepatitis B virus), flaviviruses (human hepatitis C virus) and herpesviruses (human cytomegalovirus).

This invention further relates to a means and method for measuring the replication fitness of HIV-1 that exhibits reduced drug susceptibility to reverse transcriptase inhibitors and protease inhibitors.

In a further embodiment of the invention , a means and methods are provided for measuring replication fitness for other classes of inhibitors of HIV-1 replication, including, but not limited to integration, virus assembly, and virus attachment and entry.

This invention relates to a means and method for identifying mutations in protease or reverse transcriptase that alter replication fitness.

In a further embodiment of the invention , a means and methods are provided for identifying mutations that alter replication fitness for other components of HIV-1 replication, including, but not limited to integration, virus assembly, and virus attachment and entry.

-49-

This invention also relates to a means and method for quantifying the affect that specific mutations in protease or reverse transcriptase have on replication fitness.

In a further embodiment of the invention , a means and method are provided for quantifying the affect that specific protease and reverse transcriptase mutations have on replication fitness in other viral genes involved in HIV-1 replication, including, but not limited to the gag, pol, and envelope genes.

This invention also relates to the high incidence of patient samples with reduced replication fitness.

This invention relates to the correlation between reduced drug susceptibility and reduced replication fitness.

This invention further relates to the occurrence of viruses with reduced fitness in patients receiving protease inhibitor and/or reverse transcriptase inhibitor treatment.

This invention further relates to the incidence of patient samples with reduced replication fitness in which the reduction in fitness is due to altered protease processing of the gag polyprotein (p55).

This invention further relates to the incidence of protease mutations in patient samples that exhibit low, moderate or normal (wildtype) replication fitness.

This invention further relates to protease mutations that are frequently observed, either alone or in combination, in viruses that exhibit reduced replication capacity.

5      This invention also relates to the incidence of patient samples with reduced replication fitness in which the reduction in fitness is due to altered reverse transcriptase activity. This invention relates to the occurrence of viruses with reduced replication fitness in

10     patients failing antiretroviral drug treatment. This invention further relates to a means and method for using replication fitness measurements to guide the treatment of HIV-1. This invention further relates to a means and method for using replication fitness measurements to guide

15     the treatment of patients failing antiretroviral drug treatment. This invention further relates to the means and methods for using replication fitness measurements to guide the treatment of patients newly infected with HIV-1.

20     This invention, provides the means and methods for using replication fitness measurements to guide the treatment of viral diseases, including, but not limited to HIV-1, hepadnaviruses (human hepatitis B virus), flaviviruses (human hepatitis C virus) and herpesviruses (human

25     cytomegalovirus).

In a further embodiment, the invention provides a method for determining replication capacity for a patient's virus comprising:

30

(a)     introducing a resistance test vector comprising a patient derived segment and an indicator gene into a host cell;

    (b)    culturing the host cell from (a);

    (c)    harvesting viral particles from step (b) and infecting target host cells;

5

    (d)    measuring expression of the indicator gene in the target host cell, wherein the expression of the indicator gene is dependent upon the patient-derived segment;

10    (e)    comparing the expression of the indicator gene from (d) with the expression of the indicator gene measured when steps (a) through (d) are carried out in a control resistance test vector; and

15

    (f)    normalizing the expression of the indicator gene by measuring an amount of virus in step (c).

20    As used herein, "patient-derived segment" encompasses segments derived from human and various animal species. Such species include, but are not limited to chimpanzees, horses, cattles, cats and dogs.

25    Patient-derived segments can also be incorporated into resistance test vectors using any of several alternative cloning techniques as set forth in detail in US Patent Number 5,837,464 (International Publication Number WO 97/27319) which is hereby incorporated by reference. For 30    example, cloning via the introduction of class II restriction sites into both the plasmid backbone and the patient-derived segments or by uracil DNA glycosylase primer cloning.

The patient-derived segment may be obtained by any method
of molecular cloning or gene amplification, or
modifications thereof, by introducing patient sequence
acceptor sites, as described below, at the ends of the
5      patient-derived segment to be introduced into the
resistance test vector.     For example, in a gene
amplification method such as PCR, restriction sites
corresponding to the patient-sequence acceptor sites can
be incorporated at the ends of the primers used in the PCR
10     reaction.     Similarly, in a molecular cloning method such
as cDNA cloning, said restriction sites can be
incorporated at the ends of the primers used for first or
second strand cDNA synthesis, or in a method such as
primer-repair of DNA, whether cloned or uncloned DNA, said
15     restriction sites can be incorporated into the primers
used for the repair reaction.     The patient sequence
acceptor sites and primers are designed to improve the
representation of patient-derived segments.     Sets of
resistance test vectors having designed patient sequence
20     acceptor sites provide representation of patient-derived
segments that may be underrepresented in one resistance
test vector alone.

"Resistance test vector" means one or more vectors which
25     taken together contain DNA comprising a patient-derived
segment and an indicator gene.     Resistance test vectors
are prepared as described in US Patent Number 5,837,464
(International Publication Number WO 97/27319), which is
hereby incorporated by reference, by introducing patient
30     sequence acceptor sites, amplifying or cloning
patient-derived segments and inserting the amplified or
cloned sequences precisely into indicator gene viral

vectors at the patient sequence acceptor sites.
Alternatively, a resistance test vector (also referred to
as a resistance test vector system) is prepared by
introducing patient sequence acceptor sites into a
packaging vector, amplifying or cloning patient-derived
segments and inserting the amplified or cloned sequences
precisely into the packaging vector at the patient
sequence acceptor sites and co-transfecting this packaging
vector with an indicator gene viral vector.

"Indicator or indicator gene," as described in US Patent
Number 5,837,464 (International Publication Number WO
97/27319) refers to a nucleic acid encoding a protein, DNA
or RNA structure that either directly or through a
reaction gives rise to a measurable or noticeable aspect,
e.g. a color or light of a measurable wavelength or in the
case of DNA or RNA used as an indicator a change or
generation of a specific DNA or RNA structure. Preferred
examples of an indicator gene is the E. coli lacZ gene
which encodes beta-galactosidase, the luc gene which
encodes luciferase either from, for example, Photonis
pyralis (the firefly) or Renilla reniformis (the sea
pansy), the E. coli phoA gene which encodes alkaline
phosphatase, green fluorescent protein and the bacterial
CAT gene which encodes chloramphenicol acetyltransferase.
The indicator or indicator gene may be functional or
non-functional as described in US Patent Number 5,837,464
(International Publication Number WO 97/27319).

The phenotypic drug susceptibility and resistance tests of
this invention may be carried out in one or more host
cells as described in US Patent Number 5,837,464

(International Publication Number WO 97/27319) which is incorporated herein by reference. Viral drug susceptibility is determined as the concentration of the anti-viral agent at which a given percentage of indicator

5     gene expression is inhibited (e.g. the IC50 for an anti-viral agent is the concentration at which 50% of indicator gene expression is inhibited). A standard curve for drug susceptibility of a given anti-viral drug can be developed for a viral segment that is either a standard

10     laboratory viral segment or from a drug-naive patient (i.e. a patient who has not received any anti-viral drug) using the method described in the aforementioned patent. Correspondingly, viral drug resistance is a decrease in viral drug susceptibility for a given patient compared to

15     such a given standard or when making one or more sequential measurements in the same patient over time, as determined by decreased susceptibility in virus from later time points compared to that from earlier time points.

20     The antiviral drugs being added to the test system are added at selected times depending upon the target of the antiviral drug. For example, in the case of HIV protease inhibitors, including saquinavir, ritonavir, indinavir, nelfinavir and amprenavir, they are added to packaging

25     host cells at the time of or shortly after their transfection with a resistance test vector, at an appropriate range of concentrations. HIV reverse transcriptase inhibitors, including AZT, ddI, ddC, d4T, 3TC, abacavir, nevirapine, delavirdine and efavirenz are

30     added to target host cells at the time of or prior to infection by the resistance test vector viral particles, at an appropriate range of concentration. Alternatively,

the antiviral drugs may be present throughout the assay. The test concentration is selected from a range of concentrations which is typically between about $8 \times 10^{-6}$ $\mu$M and about 2mM and more specifically for each of the following drugs: saquinavir, indinavir, nelfinavir and amprenavir, from about $2.3 \times 10^{-5}$ $\mu$M to about 1.5 $\mu$M and ritonavir, from about $4.5 \times 10^{-5}$ $\mu$M to about 3 $\mu$M.

In another embodiment of this invention, a candidate PRI antiretroviral compound is tested in the phenotypic drug susceptibility and resistance test using the resistance test vector comprising PR having a mutation at codon 88 to a serine. The candidate antiviral compound is added to the test system at an appropriate range of concentrations and at the transfection step. Alternatively, more than one candidate antiviral compound may be tested or a candidate antiviral compound may be tested in combination with an approved antiviral drug such as AZT, ddI, ddC, d4T, 3TC, abacavir, delavirdine, nevirapine, efavirenz, saquinavir, ritonavir, indinavir, nelfinavir, amprenavir, or a compound which is undergoing clinical trials such as adefovir and ABT-378. The effectiveness of the candidate antiviral will be evaluated by measuring the expression or inhibition of the indicator gene. In another aspect of this embodiment, the drug susceptibility and resistance test may be used to screen for viral mutants. Following the identification of mutants resistant to either known antiretrovirals or candidate antiretrovirals the resistant mutants are isolated and the DNA is analyzed. A library of viral resistant mutants can thus be assembled enabling the screening of candidate PRI antiretrovirals, alone or in combination. This will enable one of ordinary skill to

-56-

identify effective PRI antiretrovirals and design effective therapeutic regimens.

In another embodiment of this invention, a method of assessing the effectiveness of protease antiretroviral therapy of an HIV-infected patient is provided comprising:
(a) collecting a biological sample from the HIV-infected patient;
(b) evaluating whether the biological sample contains nucleic acid encoding HIV protease having a mutation at codon 82 or codon 90; and
(c) determining changes in susceptibility to protease inhibitors.

In another embodiment of this invention, the method is provided, wherein step (c) determines changes in susceptibility to saquinavir.

In another embodiment of this invention, the method is provided , wherein the mutation at codon 82 codes for alanine (A), phenylalanine (F), serine (S), or threonine (T).

In another embodiment of this invention, the method is provided , wherein the mutation at codon 82 is a substitution of alanine (A), phenylalanine (F), serine (S), or threonine (T) for valine(V).

In another embodiment of this invention, the method is provided, wherein the mutation at codon 90 codes for methionine (M).

In another embodiment of this invention, the method is provided , wherein the mutation at codon 90 is a substitution of methionine (M) for leucine (L).

5    In another embodiment of this invention, a method for evaluating the biological effectiveness of a candidate HIV protease antiretroviral drug compound is provided comprising:

(a)  introducing a resistance test vector comprising
10   a patient-derived segment having nucleic acid encoding HIV protease with a mutation at codon 82 or codon 90 and an indicator gene into a host cell;

(b)  culturing the host cell from step (a);

(c)  measuring the indicator gene in a target host
15   cell; and

(d)  comparing the measurement of the indicator gene from step (c) with the measurement of the indicator gene measured when steps (a) - (c) are carried out in the absence of the candidate antiretroviral drug compound;
20   wherein  a  test  concentration  of  the  candidate antiretroviral drug compound is present at steps (a) - (c); at steps (b) - (c); or at step (c).

In another embodiment of this invention, a resistance test
25   vector comprising an HIV patient-derived segment further comprising protease having a mutation at codon 82 or codon 90 and an indicator gene, wherein the expression of the indicator gene is dependent upon the patient-derived segment.

30

In another embodiment of this invention, the resistance test vector is provided , wherein the patient-derived

segment having a mutation at codon 82 codes for alanine (A), phenylalanine (F), serine (S), or threonine (T).

In another embodiment of this invention, the resistance test vector of is provided , wherein the patient-derived segment having a mutation at codon 82 is a substitution of alanine (A), phenylalanine (F), serine (S), or threonine (T) for valine(V).

In another embodiment of this invention, the resistance test vector is provided , wherein the patient-derived segment having a mutation at codon 90 codes for methionine (M).

In yet another embodiment of this invention, the resistance test vector is provided, wherein the patient-derived segment having a mutation at codon 90 is a substitution of methionine (M) for leucine (L).

In another embodiment of this invention, a method for determining replication capacity for a patient's virus is provided comprising:

(a) introducing a resistance test vector comprising a patient-derived segment and an indicator gene into a host cell;

(b) culturing the host cell from (a);

(c) harvesting viral particles from step (b) and infecting target host cells;

(d) measuring expression of the indicator gene in the target host cell, wherein the expression of the indicator gene is dependent upon the patient-derived segment; and

(e) comparing the expression of the indicator gene from (d) with the expression of the indicator gene measured when steps (a) through (d) are carried out in a control resistance test vector.

5

In another embodiment of this invention, the method further comprises the step of:

(f) normalizing the expression of the indicator gene by measuring an amount of virus in step (c).

10

In another embodiment of this invention, the method is provided wherein the patient-derived segment comprises nucleic acid encoding HIV integrase having a mutation at codon 66.

15

In another embodiment of this invention, the method is provided wherein the patient-derived segment comprises nucleic acid encoding HIV integrase having a mutation at codon 154.

20

In another embodiment of this invention, the method is provided wherein the patient-derived segment comprises nucleic acid encoding HIV integrase having mutations at codon 66 and codon 153.

25

In another embodiment of this invention, the method is provided wherein the patient-derived segment comprises nucleic acid encoding HIV integrase having mutations at codon 66 and codon 154.

30

- 60 -

In another embodiment of this invention, a method is provided of assessing the effectiveness of protease antiretroviral therapy of an HIV-infected patient comprising:

(a) collecting a biological sample from the HIV-infected patient;

(b) evaluating whether the biological sample contains nucleic acid encoding HIV protease having a mutation at codon 82 and a secondary mutation at codons selected from the group consisting of 73, 55, 48, 20, 43, 53, 90, 13, 84, 23, 33, 74, 32, 39, 60, 36, and 35, or a mutation at codon 90 and a secondary mutation at codons selected from the group consisting of 53, 95, 54, 84, 82, 46, 13, 74, 55, 85, 20, 72, 62, 66, 84, 48, 33, 73, 71, 64, 93, 23, 58, and 36; and

(c) determining a change in susceptibility to a protease inhibitor.

In another embodiment of this invention, the above method is provided of assessing the effectiveness of protease antiretroviral therapy, wherein the mutation at codon 82 is a substitution of alanine (A), phenylalanine (F), serine (S), or threonine (T) for valine(V) and the mutation at codon 90 is a substitution of methionine (M) for leucine (L).

In another embodiment of this invention, the above method is provided of assessing the effectiveness of protease antiretroviral therapy, wherein the protease inhibitor is selected from the group consisting of indinavir, amprenavir, and saquinavir.

The structure, life cycle and genetic elements of the viruses which could be tested in the drug susceptibility and resistance test of this invention would be known to one of ordinary skill in the art. It is useful to the practice of this invention, for example, to understand the life cycle of a retrovirus, as well as the viral genes required for retrovirus rescue and infectivity. Retrovirally infected cells shed a membrane virus containing a diploid RNA genome. The virus, studded with an envelope glycoprotein (which serves to determine the host range of infectivity), attaches to a cellular receptor in the plasma membrane of the cell to be infected. After receptor binding, the virus is internalized and uncoated as it passes through the cytoplasm of the host cell. Either on its way to the nucleus or in the nucleus, the reverse transcriptase molecules resident in the viral core drive the synthesis of the double-stranded DNA provirus, a synthesis that is primed by the binding of a tRNA molecule to the genomic viral RNA. The double-stranded DNA provirus is subsequently integrated in the genome of the host cell, where it can serve as a transcriptional template for both mRNAs encoding viral proteins and virion genomic RNA, which will be packaged into viral core particles. On their way out of the infected cell, core particles move through the cytoplasm, attach to the inside of the plasma membrane of the newly infected cell, and bud, taking with them tracts of membrane containing the virally encoded envelope glycoprotein gene product. This cycle of infection - reverse transcription, transcription, translation, virion assembly, and budding - repeats itself

over and over again as infection spreads.

The viral RNA and, as a result, the proviral DNA encode several cis-acting elements that are vital to the successful completion of the viral lifecycle. The virion RNA carries the viral promoter at its 3' end. Replicative acrobatics place the viral promoter at the 5' end of the proviral genome as the genome is reverse transcribed. Just 3' to the 5' retroviral LTR lies the viral packaging site. The retroviral lifecycle requires the presence of virally encoded transacting factors. The viral-RNA-dependent DNA polymerase (*pol*)-reverse transcriptase is also contained within the viral core and is vital to the viral life cycle in that it is responsible for the conversion of the genomic RNA to the integrative intermediate proviral DNA. The viral envelope glycoprotein, *env*, is required for viral attachment to the uninfected cell and for viral spread. There are also transcriptional *trans*-activating factors, so called transactivators, that can serve to modulate the level of transcription of the integrated parental provirus. Typically, replication-competent (non-defective) viruses are self-contained in that they encode all of these trans-acting factors. Their defective counterparts are not self-contained.

In the case of a DNA virus, such as a hepadnavirus, understanding the life cycle and viral genes required for infection is useful to the practice of this invention. The process of HBV entry has not been well defined. Replication of HBV uses an RNA intermediate template. In the infected cell the first step in replication is the

conversion of the asymmetric relaxed circle DNA (rc-DNA) to covalently closed circle DNA (cccDNA). This process, which occurs within the nucleus of infected liver cells, involves completion of the DNA positive-strand synthesis and ligation of the DNA ends. In the second step, the cccDNA is transcribed by the host RNA polymerase to generate a 3.5 kB RNA template (the pregenome). This pregenome is complexed with protein in the viral core. The third step involves the synthesis of the first negative-sense DNA strand by copying the pregenomic RNA using the virally encoded P protein reverse transcriptase. The P protein also serves as the minus strand DNA primer. Finally, the synthesis of the second positive-sense DNA strand occurs by copying the first DNA strand, using the P protein DNA polymerase activity and an oligomer of viral RNA as primer. The pregenome also transcribes mRNA for the major structural core proteins.

5    The following flow chart illustrates certain of the
various vectors and host cells which may be used in this
invention.  It is not intended to be all inclusive.


Vectors      Indicator      gene      cassette+Viral
10   vector(functional/nonfunctional (genomic or subgenomic)
indicator gene)

                                    ↓

Indicator  Gene  Viral  Vector  (functional/nonfunctional
indicator gene)

15

                                          + Patient sequence
                                            acceptor sites


20                                        + Patient-derived
                                            segments

                                        ↓

Resistance Test Vector
25   (patient-derived segments  +  indicator gene)
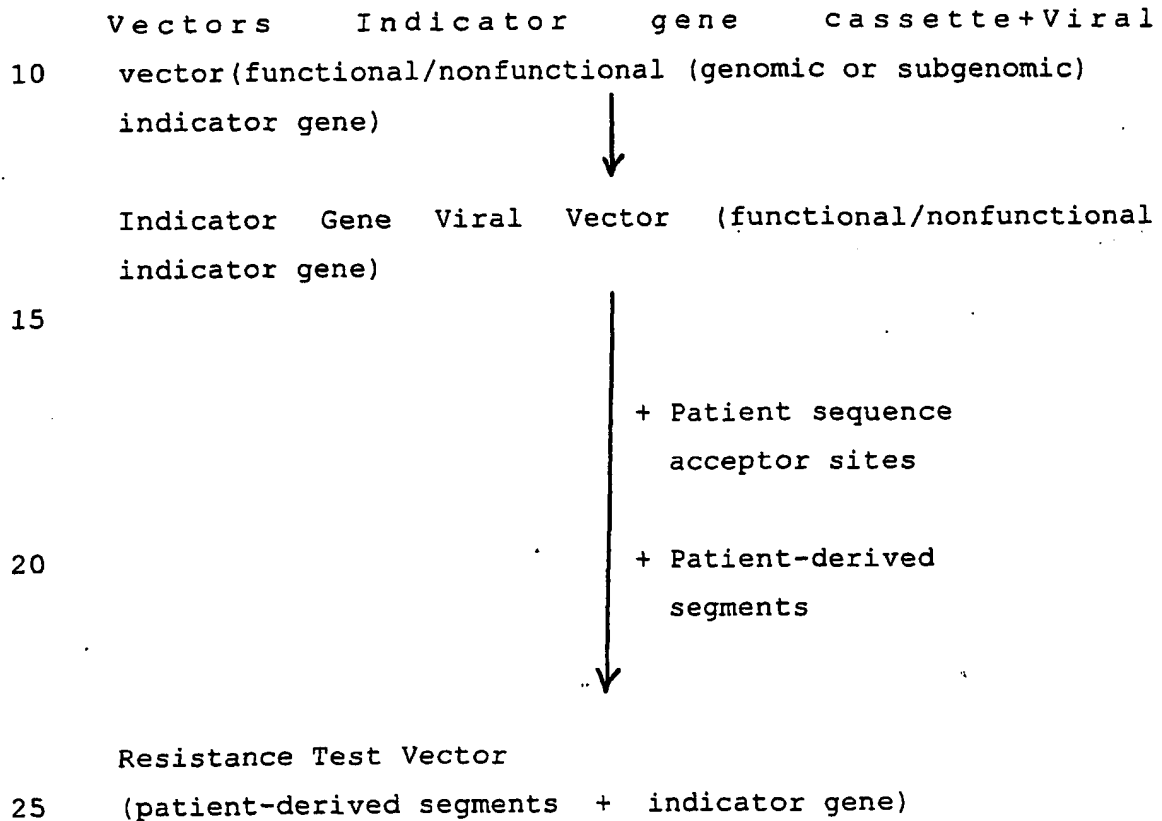


<u>Host Cells</u>


30   Packaging  Host  Cell  -  transfected  with  packaging
expression vectors


Resistance Test Vector Host Cell - a packaging host cell
transfected with a resistance test vector

35

Target  Host  Cell  -  a  host  cell  to  be  infected  by  a

-65-

resistance test vector viral particle produced by the
resistance test vector host cell

**Resistance Test Vector**

"Resistance test vector" means one or more vectors which
taken together contain DNA or RNA comprising a
patient-derived segment and an indicator gene. In the
case where the resistance test vector comprises more than
one vector the patient-derived segment may be contained in
one vector and the indicator gene in a different vector.
Such a resistance test vector comprising more than one
vector is referred to herein as a resistance test vector
system for purposes of clarity but is nevertheless
understood to be a resistance test vector. The DNA or RNA
of a resistance test vector may thus be contained in one
or more DNA or RNA molecules. In one embodiment, the
resistance test vector is made by insertion of a
patient-derived segment into an indicator gene viral
vector. In another embodiment, the resistance test vector
is made by insertion of a patient-derived segment into a
packaging vector while the indicator gene is contained in
a second vector, for example an indicator gene viral
vector. As used herein, "patient-derived segment" refers
to one or more viral segments obtained directly from a
patient using various means, for example, molecular
cloning or polymerase chain reaction (PCR) amplification
of a population of patient-derived segments using viral
DNA or complementary DNA (cDNA) prepared from viral RNA,
present in the cells (e.g. peripheral blood mononuclear
cells, PBMC), serum or other bodily fluids of infected
patients. When a viral segment is "obtained directly"
from a patient it is obtained without passage of the virus

through culture, or if the virus is cultured, then by a
minimum number of passages to essentially eliminate the
selection of mutations in culture. The term "viral
segment" refers to any functional viral sequence or viral
gene encoding a gene product (e.g., a protein) that is the
target of an anti-viral drug. The term "functional viral
sequence" as used herein refers to any nucleic acid
sequence (DNA or RNA) with functional activity such as
enhancers, promoters, polyadenylation sites, sites of
action of trans-acting factors, such as *tar* and RRE,
packaging sequences, integration sequences, or splicing
sequences. If a drug were to target more than one
functional viral sequence or viral gene product then
patient-derived segments corresponding to each said viral
gene would be inserted in the resistance test vector. In
the case of combination therapy where two or more
anti-virals targeting two different functional viral
sequences or viral gene products are being evaluated,
patient-derived segments corresponding to each functional
viral sequence or viral gene product would be inserted in
the resistance test vector. The patient-derived segments
are inserted into unique restriction sites or specified
locations, called patient sequence acceptor sites, in the
indicator gene viral vector or for example, a packaging
vector depending on the particular construction being used
as described herein.

As used herein, "patient-derived segment" encompasses
segments derived from human and various animal species.
Such species include, but are not limited to chimpanzees,
horses, cattles, cats and dogs.

5    Patient-derived segments can also be incorporated into
resistance test vectors using any of several alternative
cloning techniques.    For example, cloning via the
introduction of class II restriction sites into both the
plasmid backbone and the patient-derived segments or by
10   uracil DNA glycosylase primer cloning (refs).

The patient-derived segment may be obtained by any method
of molecular cloning or gene amplification, or
modifications thereof, by introducing patient sequence
15   acceptor sites, as described below, at the ends of the
patient-derived segment to be introduced into the
resistance test vector.    For example, in a gene
amplification method such as PCR, restriction sites
corresponding to the patient-sequence acceptor sites can
20   be incorporated at the ends of the primers used in the PCR
reaction.    Similarly, in a molecular cloning method such
as cDNA cloning, said restriction sites can be
incorporated at the ends of the primers used for first or
second strand cDNA synthesis, or in a method such as
25   primer-repair of DNA, whether cloned or uncloned DNA, said
restriction sites can be incorporated into the primers
used for the repair reaction.    The patient sequence
acceptor sites and primers are designed to improve the
representation of patient-derived segments.    Sets of
30   resistance test vectors having designed patient sequence
acceptor sites provide representation of patient-derived
segments that would be underrepresented in one resistance
test vector alone.

35   Resistance test vectors are prepared by modifying an
indicator gene viral vector (described below) by

introducing patient sequence acceptor sites, amplifying or
cloning patient-derived segments and inserting the
amplified or cloned sequences precisely into indicator
gene viral vectors at the patient sequence acceptor sites.

The resistance test vectors are constructed from
indicator gene viral vectors which are in turn derived
from genomic viral vectors or subgenomic viral vectors and
an indicator gene cassette, each of which is described
below. Resistance test vectors are then introduced into a
host cell. Alternatively, a resistance test vector (also
referred to as a resistance test vector system) is
prepared by introducing patient sequence acceptor sites
into a packaging vector, amplifying or cloning
patient-derived segments and inserting the amplified or
cloned sequences precisely into the packaging vector at
the patient sequence acceptor sites and co-transfecting
this packaging vector with an indicator gene viral vector.

In one preferred embodiment, the resistance test vector
may be introduced into packaging host cells together with
packaging expression vectors, as defined below, to produce
resistance test vector viral particles that are used in
drug resistance and susceptibility tests that are referred
to herein as a "particle-based test." In an alternative
preferred embodiment, the resistance test vector may be
introduced into a host cell in the absence of packaging
expression vectors to carry out a drug resistance and
susceptibility test that is referred to herein as a
"non-particle-based test." As used herein a "packaging
expression vector" provides the factors, such as packaging
proteins (e.g. structural proteins such as core and
envelope polypeptides), transacting factors, or genes

required by replication-defective retrovirus or
hepadnavirus. In such a situation, a
replication-competent viral genome is enfeebled in a
manner such that it cannot replicate on its own. This
means that, although the packaging expression vector can
produce the trans-acting or missing genes required to
rescue a defective viral genome present in a cell
containing the enfeebled genome, the enfeebled genome
cannot rescue itself.

*Indicator or Indicator Gene*

"Indicator or indicator gene" refers to a nucleic acid
encoding a protein, DNA or RNA structure that either
directly or through a reaction gives rise to a measurable
or noticeable aspect, e.g. a color or light of a
measurable wavelength or in the case of DNA or RNA used as
an indicator a change or generation of a specific DNA or
RNA structure. Preferred examples of an indicator gene is
the *E. coli lacZ* gene which encodes beta-galactosidase,
the *luc* gene which encodes luciferase either from, for
example, *Photonis pyralis* (the firefly) or *Renilla
reniformis* (the sea pansy), the *E. coli phoA* gene which
encodes alkaline phosphatase, green fluorescent protein
and the bacterial CAT gene which encodes chloramphenicol
acetyltransferase. Additional preferred examples of an
indicator gene are secreted proteins or cell surface
proteins that are readily measured by assay, such as
radioimmunoassay (RIA), or fluorescent activated cell
sorting (FACS), including, for example, growth factors,
cytokines and cell surface antigens (e.g. growth hormone,
Il-2 or CD4, respectively). "Indicator gene" is
understood to also include a selection gene, also referred

-70-

to as a selectable marker. Examples of suitable selectable markers for mammalian cells are dihydrofolate reductase (DHFR), thymidine kinase, hygromycin, neomycin, zeocin or *E. coli* gpt. In the case of the foregoing examples of indicator genes, the indicator gene and the patient-derived segment are discrete, i.e. distinct and separate genes. In some cases a patient-derived segment may also be used as an indicator gene. In one such embodiment in which the patient-derived segment corresponds to more than one viral gene which is the target of an anti-viral, one of said viral genes may also serve as the indicator gene. For example, a viral protease gene may serve as an indicator gene by virtue of its ability to cleave a chromogenic substrate or its ability to activate an inactive zymogen which in turn cleaves a chromogenic substrate, giving rise in each case to a color reaction. In all of the above examples of indicator genes, the indicator gene may be either "functional" or "non-functional" but in each case the expression of the indicator gene in the target cell is ultimately dependent upon the action of the patient-derived segment.

Functional Indicator Gene

In the case of a "functional indicator gene" the indicator gene may be capable of being expressed in a "packaging host cell/resistance test vector host cell" as defined below, independent of the patient-derived segment, however the functional indicator gene could not be expressed in the target host cell, as defined below, without the production of functional resistance test vector particles and their effective infection of the

target host cell.    In one embodiment of a functional
indicator gene, the indicator gene cassette, comprising
control elements and a gene encoding an indicator protein,
is inserted into the indicator gene viral vector with the
same or opposite transcriptional orientation as the native
or foreign enhancer/promoter of the viral vector.    One
example of a functional indicator gene in the case of HIV
or HBV, places the indicator gene and its promoter (a CMV
IE   enhancer/promoter)   in   the   same   or   opposite
transcriptional   orientation   as   the   HIV-LTR   or   HBV
enhancer-promoter,   respectively,   or   the   CMV   IE
enhancer/promoter associated with the viral vector.

## Non-Functional Indicator Gene

Alternatively the indicator gene, may be "non-functional"
in that the indicator gene is not efficiently expressed in
a packaging host cell transfected with the resistance test
vector, which is then referred to a resistance test vector
host cell,   until   it   is   converted   into   a   functional
indicator gene through the action of one or more of the
patient-derived segment products.    An indicator gene is
rendered   non-functional   through   genetic   manipulation
according to this invention.

1. <u>Permuted Promoter</u>   In one embodiment an indicator gene
is   rendered   non-functional   due   to   the   location   of   the
promoter, in that, although the promoter is in the same
transcriptional   orientation   as   the   indicator   gene,   it
follows rather than precedes the indicator gene coding
sequence.    This misplaced promoter is referred to as a
"permuted promoter."   In addition to the permuted promoter
the orientation of the non-functional indicator gene is

opposite to that of the native or foreign promoter/enhancer of the viral vector. Thus the coding sequence of the non-functional indicator gene can neither be transcribed by the permuted promoter nor by the viral promoters. The non-functional indicator gene and its permuted promoter is rendered functional by the action of one or more of the viral proteins. One example of a non-functional indicator gene with a permuted promoter in the case of HIV, places a T7 phage RNA polymerase promoter (herein referred to as T7 promoter) promoter in the 5' LTR in the same transcriptional orientation as the indicator gene. The indicator gene cannot be transcribed by the T7 promoter as the indicator gene cassette is positioned upstream of the T7 promoter. The non-functional indicator gene in the resistance test vector is converted into a functional indicator gene by reverse transcriptase upon infection of the target cells, resulting from the repositioning of the T7 promoter, by copying from the 5' LTR to the 3' LTR, relative to the indicator gene coding region. Following the integration of the repaired indicator gene into the target cell chromosome by HIV integrase, a nuclear T7 RNA polymerase expressed by the target cell transcribes the indicator gene. One example of a non-functional indicator gene with a – permuted promoter in the case of HBV, places an enhancer-promoter region downstream or 3' of the indicator gene both having the same transcriptional orientation. The indicator gene cannot be transcribed by the enhancer-promoter as the indicator gene cassette is positioned upstream. The non-functional indicator gene in the resistance test vector is converted into a functional indicator gene by reverse transcription and circularization of the HBV

●                    ●

5    indicator gene viral vector by the repositioning of the
     enhancer-promoter upstream relative to the indicator gene
     coding region.

     A permuted promoter may be any eukaryotic or prokaryotic
10   promoter which can be transcribed in the target host cell.
     Preferably the promoter will be small in size to enable
     insertion in the viral genome without disturbing viral
     replication.  More preferably, a promoter that is small in
     size and is capable of transcription by a single subunit
15   RNA polymerase introduced into the target host cell, such
     as a bacteriophage promoter, will be used.  Examples of
     such bacteriophage promoters and their cognate RNA
     polymerases include those of phages T7, T3 and Sp6.  A
     nuclear localization sequence (NLS) may be attached to the
20   RNA polymerase to localize expression of the RNA
     polymerase to the nucleus where they may be needed to
     transcribed the repaired indicator gene.  Such an NLS may
     be obtained from any  nuclear-transported protein such as
     the SV40 T antigen.  If a phage RNA polymerase is
25   employed, an internal ribosome entry site (IRES) such as
     the EMC virus 5' untranslated region (UTR) may be added in
     front of the indicator gene, for translation of the
     transcripts which are generally uncapped.  In the case of
     HIV, the permuted promoter itself can be introduced at any
30   position within the 5' LTR that is copied to the 3' LTR
     during reverse transcription so long as LTR function is
     not disrupted, preferably within the U5 and R portions of
     the LTR, and most preferably outside of functionally
     important and highly conserved regions of U5 and R.  In
35   the case of HBV, the permuted promoter can be placed at
     any position that does not disrupt the cis acting elements

that are necessary for HBV DNA replication. Blocking
sequences may be added at the ends of the resistance test
vector should there be inappropriate expression of the
non-functional indicator gene due to transfection
artifacts (DNA concatenation). In the HIV example of the
permuted T7 promoter given above, such a blocking sequence
may consist of a T7 transcriptional terminator, positioned
to block readthrough transcription resulting from DNA
concatenation, but not transcription resulting from
repositioning of the permuted T7 promoter from the 5' LTR
to the 3' LTR during reverse transcription.

2. Permuted Coding Region    In a second embodiment, an
indicator gene is rendered non-functional due to the
relative location of the 5' and 3' coding regions of the
indicator gene, in that, the 3' coding region precedes
rather than follows the 5' coding region. This misplaced
coding region is referred to as a "permuted coding
region." The orientation of the non-functional indicator
gene may be the same or opposite to that of the native or
foreign promoter/enhancer of the viral vector, as mRNA
coding for a functional indicator gene will be produced in
the event of either orientation. The non-functional
indicator gene and its permuted coding region is rendered
functional by the action of one or more of the
patient-derived segment products. A second example of a
non-functional indicator gene with a permuted coding
region in the case of HIV, places a 5' indicator gene
coding region with an associated promoter in the 3' LTR U3
region and a 3' indicator gene coding region in an
upstream location of the HIV genome, with each coding
region having the same transcriptional orientation as the

viral LTRs.   In both examples, the 5' and 3' coding regions may also have associated splice donor and acceptor sequences, respectively, which may be heterologous or artificial splicing signals.   The indicator gene cannot be functionally transcribed either by the associated promoter or viral promoters, as the permuted coding region prevents the formation of functionally spliced transcripts.   The non-functional indicator gene in the resistance test vector is converted into a functional indicator gene by reverse transcriptase upon infection of the target cells, resulting from the repositioning of the 5' and 3' indicator gene coding regions relative to one another, by copying of the 3' LTR to the 5' LTR.   Following transcription by the promoter associated with the 5' coding region, RNA splicing can join the 5' and 3' coding regions to produce a functional indicator gene product. One example of a non-functional indicator gene with a permuted coding region in the case of HBV, places a 3' indicator gene coding region upstream or 5' of the enhancer-promoter and the 5' coding region of the indicator gene.   The transcriptional orientation of the indicator gene 5' and 3' coding regions are identical to one another, and the same as that of the indicator gene viral vector.   However, as the indicator gene 5' and 3' coding regions are permuted in the resistance test vectors (i.e., the 5' coding region is downstream of the 3' coding region), no mRNA is transcribed which can be spliced to generate a functional indicator gene coding region. Following reverse transcription and circularization of the indicator gene viral vector, the indicator gene 3' coding region is positioned downstream or 3' to the enhancer-promoter and 5' coding regions thus permitting

5    the transcription of mRNA which can be spliced to generate
a functional indicator gene coding region.

3. _Inverted Intron_   In a third embodiment, the indicator
gene is rendered non-functional through use of an
10   "inverted intron," i.e. an intron inserted into the coding
sequence of the indicator gene with a transcriptional
orientation opposite to that of the indicator gene.   The
overall transcriptional orientation of the indicator gene
cassette including its own, linked promoter, is opposite
15   to that of the viral control elements, while the
orientation of the artificial intron is the same as the
viral control elements.   Transcription of the indicator
gene by its own linked promoter does not lead to the
production of functional transcripts as the inverted
20   intron cannot be spliced in this orientation.
Transcription of the indicator gene by the viral control
elements does, however, lead to the removal of the
inverted intron by RNA splicing, although the indicator
gene is still not functionally expressed as the resulting
25   transcript has an antisense orientation.   Following the
reverse transcription of this transcript and integration
of the resultant retroviral DNA, or the circularization of
hepadnavirus DNA, the indicator gene can be functionally
transcribed using its own linked promoter as the inverted
30   intron has been previously removed.   In this case, the
indicator gene itself may contain its own functional
promoter with the entire transcriptional unit oriented
opposite to the viral control elements.   Thus the
non-functional indicator gene is in the wrong orientation
35   to be transcribed by the viral control elements and it
cannot be functionally transcribed by its own promoter, as

5    the inverted intron cannot be properly excised by splicing. However, in the case of a retrovirus and HIV specifically and hepadnaviruses, and HBV specifically, transcription by the viral promoters (HIV LTR or HBV enhancer-promoter) results in the removal of the inverted

10   intron by splicing. As a consequence of reverse transcription of the resulting spliced transcript and the integration of the resulting provirus into the host cell chromosome or circularization of the HBV vector, the indicator gene can now be functionally transcribed by its

15   own promoter. The inverted intron, consisting of a splice donor and acceptor site to remove the intron, is preferably located in the coding region of the indicator gene in order to disrupt translation of the indicator gene. The splice donor and acceptor may be any splice

20   donor and acceptor. A preferred splice donor-receptor is the CMV IE splice donor and the splice acceptor of the second exon of the human alpha globin gene ("intron A").

### Indicator Gene Viral Vector - Construction

25   As used herein, "indicator gene viral vector" refers to a vector(s) comprising an indicator gene and its control elements and one or more viral genes. The indicator gene viral vector is assembled from an indicator gene cassette and a "viral vector," defined below. The indicator gene

30   viral vector may additionally include an enhancer, splicing signals, polyadenylation sequences, transcriptional terminators, or other regulatory sequences. Additionally the indicator gene viral vector may be functional or nonfunctional. In the event that the

35   viral segments which are the target of the anti-viral drug are not included in the indicator gene viral vector they

are provided in a second vector. An "indicator gene cassette" comprises an indicator gene and control elements. "Viral vector" refers to a vector comprising some or all of the following: viral genes encoding a gene product, control sequences, viral packaging sequences, and in the case of a retrovirus, integration sequences. The viral vector may additionally include one or more viral segments one or more of which may be the target of an anti-viral drug. Two examples of a viral vector which contain viral genes are referred to herein as an "genomic viral vector" and a "subgenomic viral vector." A "genomic viral vector" is a vector which may comprise a deletion of a one or more viral genes to render the virus replication incompetent, but which otherwise preserves the mRNA expression and processing characteristics of the complete virus. In one embodiment for an HIV drug susceptibility and resistance test, the genomic viral vector comprises the HIV *gag-pol*, *vif*, *vpr*, *tat*, *rev*, *vpu*, and *nef* genes (some, most or all of *env* may be deleted). A "subgenomic viral vector" refers to a vector comprising the coding region of one or more viral genes which may encode the proteins that are the target(s) of the anti-viral drug. In the case of HIV, a preferred embodiment is a subgenomic viral vector comprising the HIV *gag-pol* gene. In the case of HBV a preferred embodiment is a subgenomic viral vector comprising the HBV P gene. In the case of HIV, two examples of proviral clones used for viral vector construction are: HXB2 (Fisher et al., (1986) *Nature*, **320**, 367-371) and NL4-3, (Adachi et al., (1986) *J. Virol.*, **59**, 284-291). In the case of HBV, a large number of full length genomic sequences have been characterized and could be used for construction of HBV viral vectors: GenBank

5      Nos. M54923, M38636, J02203 and X59795. The viral coding
       genes   may    be    under   the    control   of    a    native
       enhancer/promoter   or   a  foreign   viral   or   cellular
       enhancer/promoter. A preferred embodiment for an HIV drug
       susceptibility  and   resistance   test,   is   to   place   the
10     genomic   or   subgenomic   viral   coding   regions   under   the
       control of the native enhancer/promoter of the HIV-LTR U3
       region or the CMV immediate-early (IE) enhancer/promoter.
       A preferred embodiment for an HBV drug susceptibility and
       resistance test, is to place the genomic or subgenomic
15     viral   coding   regions   under   the   control   of   the   CMV
       immediate-early (IE) enhancer/promoter. In the case of an
       indicator   gene   viral   vector   that   contains   one   or   more
       viral genes which are the targets or encode proteins which
       are the targets of an anti-viral drug(s) then said vector
20     contains   the   patient   sequence   acceptor   sites.      The
       patient-derived   segments   are   inserted   in   the   patient
       sequence acceptor site in the indicator gene viral vector
       which is then referred to as the resistance test vector,
       as described above.

25

       "Patient   sequence   acceptor   sites"   are   sites   in   a   vector
       for insertion of patient-derived segments and said sites
       may   be:   1)   unique   restriction   sites   introduced   by
       site-directed   mutagenesis   into   a   vector;   2)   naturally
30     occurring   unique   restriction   sites   in   the   vector;   or   3)
       selected sites into which a patient-derived segment may be
       inserted   using   alternative   cloning   methods   (e.g.   UDG
       cloning).   In one embodiment the patient sequence acceptor
       site is introduced into the indicator gene viral vector.
35     The patient sequence acceptor sites are preferably located
       within   or   near   the   coding   region   of   the   viral   protein

which is the target of the anti-viral drug. The viral
sequences used for the introduction of patient sequence
acceptor sites are preferably chosen so that no change, or
a conservative change, is made in the amino acid coding
sequence found at that position. Preferably the patient
sequence acceptor sites are located within a relatively
conserved region of the viral genome to facilitate
introduction of the patient-derived segments.
Alternatively, the patient sequence acceptor sites are
located between functionally important genes or regulatory
sequences. Patient-sequence acceptor sites may be located
at or near regions in the viral genome that are relatively
conserved to permit priming by the primer used to
introduce the corresponding restriction site into the
patient-derived segment. To improve the representation of
patient-derived segments further, such primers may be
designed as degenerate pools to accommodate viral sequence
heterogeneity, or may incorporate residues such as
deoxyinosine (I) which have multiple base-pairing
capabilities. Sets of resistance test vectors having
patient sequence acceptor sites that define the same or
overlapping restriction site intervals may be used
together in the drug resistance and susceptibility tests
to provide representation of patient-derived segments that
contain internal restriction sites identical to a given
patient sequence acceptor site, and would thus be
underrepresented in either resistance test vector alone.


***Host Cells***

The resistance test vector is introduced into a host cell.
Suitable host cells are mammalian cells. Preferred host
cells are derived from human tissues and cells which are

-81-

the principle targets of viral infection. In the case of HIV these include human cells such as human T cells, monocytes, macrophage, dendritic cells, Langerhans cells, hematopoeitic stem cells or precursor cells, and other cells. In the case of HBV, suitable host cells include hepatoma cell lines (HepG2, Huh 7), primary human hepatocytes, mammalian cells which can be- infected by pseudotyped HBV, and other cells. Human derived host cells will assure that the anti-viral drug will enter the cell efficiently and be converted by the cellular enzymatic machinery into the metabolically relevant form of the anti-viral inhibitor. Host cells are referred to herein as a "packaging host cells," "resistance test vector host cells," or "target host cells." A "packaging host cell" refers to a host cell that provides the trans-acting factors and viral packaging proteins required by the replication defective viral vectors used herein, such as the resistance test vectors, to produce resistance test vector viral particles. The packaging proteins may be provided for by the expression of viral genes contained within the resistance test vector itself, a packaging expression vector(s), or both. A packaging host cell is a host cell which is transfected with one or more packaging expression vectors and when transfected with a resistance test vector is then referred to herein as a "resistance test vector host cell" and is sometimes referred to as a packaging host cell/resistance test vector host cell. Preferred host cells for use as packaging host cells for HIV include 293 human embryonic kidney cells (293, Graham, F.L. et al., J. Gen Virol. 36: 59, 1977), BOSC23 (Pear et al., *Proc. Natl. Acad. Sci.* **90**, 8392, 1993), tsa54 and tsa201 cell lines (Heinzel et al., *J.Virol.* **62**,

 5    3738,1988), for HBV HepG2 (Galle and Theilmann, L.
      Arzheim.-Forschy *Drug Res.* (1990) **40**, 1380-1382). (Huh,
      Ueda, K et al. *Virology* *1989) **169**, 213-216). A "target
      host cell" refers to a cell to be infected by resistance
      test vector viral particles produced by the resistance
10    test vector host cell in which expression or inhibition of
      the indicator gene takes place. Preferred host cells for
      use as target host cells include human T cell leukemia
      cell lines including Jurkat (ATCC T1B-152), H9 (ATCC
      HTB-176), CEM (ATCC CCL-119), HUT78 (ATCC T1B-161), and
15    derivatives thereof.


      This invention is illustrated in the Experimental Detais
      section which follows. These sections are set forth to
      aid in an understanding of the invention but are not
20    intended to, and should not be construed to, limit in any
      way the invention as set forth in the claims which follow
      thereafter.


      **Experimental Details**
25    General Materials and Methods
      Most of the techniques used to construct vectors, and
      transfect and infect cells, are widely practiced in the
      art, and most practitioners are familiar with the standard
      resource materials that describe specific conditions and
30    procedures. However, for convenience, the following
      paragraphs may serve as a guideline.


      As used herein, "replication capacity" is defined herein
      is a measure of how well the virus replicates. This may
35    also be referred to as viral fitness. In one embodiment,
      replication capacity can be measured by evaluating the

5      ability of the virus to replicate in a single round of
       replication.

       As used herein, "control resistance test vector" is
       defined as a resistance test vector comprising a standard
10     viral sequence (for example, HXB2, PNL4-3) and an
       indicator gene.

       As used herein, "normalizing" is defined as standardizing
       the amount of the expression of indicator gene measured
15     relative to the number of viral particles giving rise to
       the expression of the indicator gene. For example,
       normalization is measured by dividing the amount of
       luciferase activity measured by the number of viral
       particles measured at the time of infection.

20

       "Plasmids" and "vectors" are designated by a lower case p
       followed by letters and/or numbers. The starting plasmids
       herein are either commercially available, publicly
       available on an unrestricted basis, or can be constructed
25     from available plasmids in accord with published
       procedures. In addition, equivalent plasmids to those
       described are known in the art and will be apparent to the
       ordinarily skilled artisan.

30     Construction of the vectors of the invention employs
       standard ligation and restriction techniques which are
       well understood in the art (see Ausubel et al., (1987)
       Current Protocols in Molecular Biology, Wiley -
       Interscience or Maniatis et al., (1992) in Molecular
35     Cloning: A laboratory Manual, Cold Spring Harbor
       Laboratory, N.Y.). Isolated plasmids, DNA sequences, or

5      synthesized oligonucleotides are cleaved, tailored, and
       religated in the form desired. The sequences of all DNA
       constructs incorporating synthetic DNA were confirmed by
       DNA sequence analysis (Sanger et al. (1977) Proc. Natl.
       Acad. Sci. 74, 5463-5467).

10

       "Digestion" of DNA refers to catalytic cleavage of the DNA
       with a restriction enzyme that acts only at certain
       sequences, restriction sites, in the DNA. The various
       restriction enzymes used herein are commercially available

15     and their reaction conditions, cofactors and other
       requirements are known to the ordinarily skilled artisan.
       For analytical purposes, typically 1 µg of plasmid or DNA
       fragment is used with about 2 units of enzyme in about 20
       µl of buffer solution. Alternatively, an excess of

20     restriction enzyme is used to insure complete digestion of
       the DNA substrate. Incubation times of about one hour to
       two hours at about 37°C are workable, although variations
       can be tolerated. After each incubation, protein is
       removed by extraction with phenol/chloroform and the

25     nucleic acid recovered from aqueous fractions by
       precipitation with ethanol. If desired, size separation
       of the cleaved fragments may be performed by
       polyacrylamide gel or agarose gel electrophoresis using
       standard techniques. A general description of size

30     separations is found in Methods of Enzymology 65:499-560
       (1980).

       Restriction cleaved fragments may be blunt ended by
       treating with the large fragment of E. coli DNA polymerase

35     I (Klenow) in the presence of the four deoxynucleotide
       triphosphates (dNTPs) using incubation times of about 15

5      to 25 minutes at 20°C in 50 mM Tris (pH 7.6) 50 mM NaCl, 6
       mM $MgCl_2$, 6 mM DTT and 5-10 mM dNTPs. The Klenow fragment
       fills in at 5' sticky ends but chews back protruding 3'
       single strands, even though the four dNTPs are present.
       If desired, selective repair can be performed by supplying
10     only one of the dNTPs, or with selected dNTPs, within the
       limitations dictated by the nature of the sticky ends.
       After treatment with Klenow, the mixture is extracted with
       phenol/chloroform and ethanol precipitated.    Treatment
       under appropriate conditions with S1 nuclease or Bal-31
15     results in hydrolysis of any single-stranded portion.

       Ligations are performed in 15-50 µl volumes under the
       following standard conditions and temperatures:  20  mM
       Tris-Cl pH 7.5, 10 mM $MgCl_2$, 10 mM DTT, 33 mg/ml BSA, 10
20     mM- 50 mM NaCl, and either 40 µM ATP, 0.01-0.02 (Weiss)
       units T4 DNA ligase at 0°C (for "sticky end" ligation) or
       1mM ATP, 0.3 - 0.6 (Weiss) units T4 DNA ligase at 14°C (for
       "blunt  end"  ligation).    Intermolecular  "sticky  end"
       ligations are usually performed at 33-100 µg/ml total DNA
25     concentrations   (5-100   mM   total   end   concentration).
       Intermolecular blunt end ligations (usually employing a
       10-30 fold molar excess of linkers) are performed at 1µM
       total ends concentration.

30     "Transient expression" refers to unamplified expression
       within about one day to two weeks of transfection.  The
       optimal time for transient expression of a particular
       desired heterologous gene may vary depending on several
       factors including, for example, any transacting factors
35     which may be employed, translational control mechanisms
       and the host cell.  Transient expression occurs when the

5     particular plasmid that has been transfected functions,
i.e., is transcribed and translated. During this time the
plasmid DNA which has entered the cell is transferred to
the nucleus. The DNA is in a nonintegrated state, free
within the nucleus. Transcription of the plasmid taken up
10     by the cell occurs during this period. Following
transfection the plasmid DNA may become degraded or
diluted by cell division. Random integration within the
cell chromatin occurs.

15     In general, vectors containing promoters and control
sequences which are derived from species compatible with
the host cell are used with the particular host cell.
Promoters suitable for use with prokaryotic hosts
illustratively include the beta-lactamase and lactose
20     promoter systems, alkaline phosphatase, the tryptophan
(trp) promoter system and hybrid promoters such as tac
promoter. However, other functional bacterial promoters
are suitable. In addition to prokaryotes, eukaryotic
microbes such as yeast cultures may also be used.
25     Saccharomyces cerevisiae, or common baker's yeast is the
most commonly used eukaryotic microorganism, although a
number of other strains are commonly available. Promoters
controlling transcription from vectors in mammalian host
cells may be obtained from various sources, for example,
30     the genomes of viruses such as: polyoma, simian virus 40
(SV40), adenovirus, retroviruses, hepatitis B virus and
preferably cytomegalovirus, or from heterologous mammalian
promoters, e.g. β-actin promoter. The early and late
promoters of the SV 40 virus are conveniently obtained as
35     an SV40 restriction fragment that also contains the SV40
viral origin of replication. The immediate early promoter

5      of the human cytomegalovirus is conveniently obtained as a HindIII E restriction fragment.  Of course, promoters from the host cell or related species also are useful herein.

     The vectors used herein may contain a selection gene, also

10      termed a selectable marker.  A selection gene encodes a protein, necessary for the survival or growth of a host cell transformed with the vector.  Examples of suitable selectable markers for mammalian cells include the dihydrofolate reductase gene (DHFR), the ornithine

15      decarboxylase gene, the multi-drug resistance gene (mdr), the adenosine deaminase gene, and the glutamine synthase gene.  When such selectable markers are successfully transferred into a mammalian host cell, the transformed mammalian host cell can survive if placed under selective

20      pressure.  There are two widely used distinct categories of selective regimes.  The first category is based on a cell's metabolism and the use of a mutant cell line which lacks the ability to grow independent of a supplemented media.  The second category is referred to as dominant

25      selection which refers to a selection scheme used in any cell type and does not require the use of a mutant cell line.  These schemes typically use a drug to arrest growth of a host cell.  Those cells which have a novel gene would express a protein conveying drug resistance and would

30      survive the selection.  Examples of such dominant selection use the drugs neomycin (Southern and Berg (1982) J. Molec. Appl. Genet. 1, 327), mycophenolic acid (Mulligan and Berg (1980) Science 209, 1422), or hygromycin (Sugden et al. (1985) Mol. Cell. Biol. 5,

35      410-413).  The three examples given above employ bacterial genes under eukaryotic control to convey resistance to the

5      appropriate drug neomycin (G418 or genticin), xgpt
       (mycophenolic acid) or hygromycin, respectively.

       "Transfection" means introducing DNA into a host cell so
       that the DNA is expressed, whether functionally expressed
10     or otherwise; the DNA may also replicate either as an
       extrachromosomal element or by chromosomal integration.
       Unless otherwise provided, the method used herein for
       transfection of the host cells is the calcium phosphate
       co-precipitation method of Graham and van der Eb (1973)
15     Virology 52, 456-457.    Alternative methods for
       transfection are electroporation, the DEAE-dextran method,
       lipofection and biolistics (Kriegler (1990) Gene Transfer
       and Expression: A Laboratory Manual, Stockton Press).

20     Host cells may be transfected with the expression vectors
       of the present invention and cultured in conventional
       nutrient media modified as is appropriate for inducing
       promoters, selecting transformants or amplifying genes.
       Host cells are cultured in F12:DMEM (Gibco) 50:50 with
25     added glutamine.   The culture conditions, such as
       temperature, pH and the like, are those previously used
       with the host cell selected for expression, and will be
       apparent to the ordinarily skilled artisan.

30     The following examples merely illustrate the best mode now
       known for practicing the invention, but should not be
       construed to limit the invention.  All publications and
       patent applications cited in this specification are herein
       incorporated by reference in their entirety as if each
35     individual publication or patent application were
       specifically and individually indicated to be incorporated

5    by reference.

## EXAMPLE 1

### Phenotypic Drug Susceptibility and Resistance Test Using Resistance Test Vectors

10    Phenotypic drug susceptibility and resistance tests are carried out using the means and methods described in US Patent Number 5,837,464 (International Publication Number WO 97/27319) which is hereby incorporated by reference.

15    In these experiments patient-derived segment(s) corresponding to the HIV protease and reverse transcriptase coding regions were either patient-derived segments amplified by the reverse transcription-polymerase chain reaction method (RT-PCR) using viral RNA isolated
20    from viral particles present in the serum of HIV-infected individuals or were mutants of wild type HIV-1 made by site directed mutagenesis of a parental clone of resistance test vector DNA. Isolation of viral RNA was performed using standard procedures (e.g. RNAgents Total
25    RNA Isolation System, Promega, Madison WI or RNAzol, Tel-Test, Friendswood, TX). The RT-PCR protocol was divided into two steps. A retroviral reverse transcriptase [e.g. Moloney MuLV reverse transcriptase (Roche Molecular Systems, Inc., Branchburg, NJ), or avian
30    myeloblastosis virus (AMV) reverse transcriptase, (Boehringer Mannheim, Indianapolis, IN)] was used to copy viral RNA into cDNA. The cDNA was then amplified using a thermostable DNA polymerase [e.g. Taq (Roche Molecular Systems, Inc., Branchburg, NJ), Tth (Roche Molecular
35    Systems, Inc., Branchburg, NJ), PrimeZyme (isolated from Thermus brockianus, Biometra, Gottingen, Germany)] or a

combination of thermostable polymerases as described for
the performance of "long PCR" (Barnes, W.M., (1994) Proc.
Natl. Acad. Sci, USA 91, 2216-2220) [e.g. Expand High
Fidelity PCR System (Taq + Pwo), (Boehringer Mannheim.
Indianapolis, IN) OR GeneAmp XL PCR kit (Tth + Vent),
(Roche Molecular Systems, Inc., Branchburg, NJ)].

PCR6 (Table 5, #1) is used for reverse transcription of
viral RNA into cDNA. The primers, ApaI primer (PDSApa,
Table 5, #2) and AgeI primer (PDSAge, Table 5, #3) used to
amplify the "test" patient-derived segments contained
sequences resulting in ApaI and AgeI recognition sites
being introduced into both ends of the PCR product,
respectively.
Resistance test vectors incorporating the "test"
patient-derived segments were constructed as described in
US Patent Number 5,837,464 (International Publication
Number WO 97/27319) (see Fig. 1) using an amplified DNA
product of 1.5 kB prepared by RT-PCR using viral RNA as a
template and oligonucleotides PCR6 (#1), PDSApa (#2) and
PDSAge (#3) as primers, followed by digestion with ApaI
and AgeI or the isoschizomer PinAl. To ensure that the
plasmid DNA corresponding to the resultant resistance test
vector comprises a representative sample of the HIV viral
quasi-species present in the serum of a given patient,
many (>100) independent E. coli transformants obtained in
the construction of a given resistance test vector were
pooled and used for the preparation of plasmid DNA.

A packaging expression vector encoding an amphotrophic
MuLV 4070A env gene product enables production in a
resistance test vector host cell of resistance test vector

●                    ●

-91-

5      viral particles which can efficiently infect human target
       cells.    Resistance test vectors encoding all HIV genes
       with  the  exception  of  env  were  used  to  transfect  a
       packaging host cell (once transfected the host cell is
       referred to as a resistance test vector host cell).   The
10     packaging expression vector which encodes the amphotrophic
       MuLV  4070A  env  gene  product  is  used  with  the  resistance
       test  vector  to  enable  production  in  the  resistance  test
       vector host cell of infectious pseudotyped resistance test
       vector viral particles.

15

       Resistance  tests  performed  with  resistance  test  vectors
       were  carried  out  using  packaging  host  and  target  host
       cells  consisting  of  the  human  embryonic  kidney  cell  line
       293  (Cell  Culture  Facility,  UC  San  Francisco,  SF,  CA)  or
20     the   Jurkat   leukemic   T-cell   line   (Arthur   Weiss,   UC   San
       Francisco, SF, CA).

       Resistance  tests  were  carried  out  with  resistance  test
       vectors  using  two  host  cell  types.    Resistance  test  vector
25     viral  particles  were  produced  by  a  first  host  cell  (the
       resistance  test  vector  host  cell)  that  was  prepared  by
       transfecting  a  packaging  host  cell  with  the  resistance
       test  vector  and  the  packaging  expression  vector.    The
       resistance  test  vector  viral  particles  were  then  used  to
30     infect  a  second  host  cell  (the  target  host  cell)  in  which
       the  expression  of  the  indicator  gene  is  measured  (see  Fig.
       2).

       The   resistance   test   vectors   containing   a   functional
35     luciferase  gene  cassette  were  constructed  and  host  cells
       were  transfected  with  the  resistance  test  vector  DNA.    The

●

●

resistant test vectors contained patient-derived reverse transcriptase and protease DNA sequences that encode proteins which were either susceptible or resistant to the antiretroviral agents, such as nucleoside reverse transcriptase inhibitors, non-nucleoside reverse transcriptase inhibitors and protease inhibitors. The resistance test vector viral particles produced by transfecting the resistance test vector DNA into host cells, either in the presence or absence of protease inhibitors, were used to infect target host cells grown either in the absence of NRTI or NNRTI or in the presence of increasing concentrations of the drug. Luciferase activity in infected target host cells in the presence of drug was compared to the luciferase activity in infected target host cells in the absence of drug. Drug resistance was measured as the concentration of drug required to inhibit by 50% the luciferase activity detected in the absence of drug (inhibitory concentration 50%, IC50 ). The IC50 values were determined by plotting percent drug inhibition vs. $\log_{10}$ drug concentration.

Host cells were seeded in 10-cm-diameter dishes and were transfected one day after plating with resistance test vector plasmid DNA and the envelope expression vector. Transfections were performed using a calcium-phosphate co-precipitation procedure. The cell culture media containing the DNA precipitate was replaced with fresh medium, from one to 24 hours, after transfection. Cell culture media containing resistance test vector viral particles was harvested one to four days after transfection and was passed through a 0.45-mm filter before being stored at $^-80^oC$. HIV capsid protein (p24)

levels in the harvested cell culture media were determined by an EIA method as described by the manufacturer (SIAC; Frederick, MD). Before infection, target cells (293 and 293/T) were plated in cell culture media. Control infections were performed using cell culture media from mock transfections (no DNA) or transfections containing the resistance test vector plasmid DNA without the envelope expression plasmid. One to three or more days after infection the media was removed and cell lysis buffer (Promega) was added to each well. Cell lysates were assayed for luciferase activity. The inhibitory effect of the drug was determined using the following equation:

% luciferase inhibition = [1 - (RLUluc [drug]    RLUluc)] x 100

where RLUluc [drug] is the relative light unit of luciferase activity in infected cells in the presence of drug and RLUluc is the Relative Light Unit of luciferase activity in infected cells in the absence of drug. IC50 values were obtained from the sigmoidal curves that were generated from the data by plotting the percent inhibition of luciferase activity vs. the log₁₀ drug concentration. Examples of drug inhibition curves are shown in (Fig. 3).

## EXAMPLE 2

**An in vitro Assay Using Resistance Test Vectors And Site Directed Mutants To Correlate Phenotypes And Genotypes Associated With HIV Drug Susceptibility And Resistance**

Phenotypic susceptibility analysis of patient HIV samples Resistance test vectors are constructed as described in

example 1. Resistance test vectors, or clones derived from the resistance test vector pools, are tested in a phenotypic assay to determine accurately and quantitatively the level of susceptibility to a panel of anti-retroviral drugs. This panel of anti-retroviral drugs may comprise members of the classes known as nucleoside-analog reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), and protease inhibitors (PRIs). The panel of drugs can be expanded as new drugs or new drug targets become available. An IC50 is determined for each resistance test vector pool for each drug tested. The pattern of susceptibility to all of the drugs tested is examined and compared to known patterns of susceptibility.

A patient sample can be further examined for genotypic changes correlated with the pattern of susceptibility observed.

**Genotypic analysis of patient HIV samples**

Resistance test vector DNAs, either pools or clones, are analyzed by any of the genotyping methods described in Example 1. In one embodiment of the invention, patient HIV sample sequences are determined using viral RNA purification, RT/PCR and ABI chain terminator automated sequencing. The sequence that is determined is compared to control sequences present in the database or is compared to a sample from the patient prior to initiation of therapy, if available. The genotype is examined for sequences that are different from the control or pre-treatment sequence and correlated to the observed phenotype.

**Phenotypic susceptibility analysis of site directed mutants**

Genotypic changes that are observed to correlate with changes in phenotypic patterns of drug susceptibility are evaluated by construction of resistance test vectors containing the specific mutation on a defined, wild-type (drug susceptible) genetic background. Mutations may be incorporated alone and/or in combination with other mutations that are thought to modulate the susceptibility of HIV to a certain drug or class of drugs. Mutations are introduced into the resistance test vector through any of the widely known methods for site-directed mutagenesis. In one embodiment of this invention the mega-primer PCR method for site-directed mutagenesis is used. A resistance test vector containing the specific mutation or group of mutations are then tested using the phenotypic susceptibility assay described above and the susceptibility profile is compared to that of a genetically defined wild-type (drug susceptible) resistance test vector which lacks the specific mutations. Observed changes in the pattern of phenotypic susceptibility to the antiretroviral drugs tested are attributed to the specific mutations introduced into the resistance test vector.

**EXAMPLE 3**

**Using Resistance Test Vectors To Correlate Genotypes And Phenotypes Associated With Changes in PRI Drug Susceptibility in HIV.**

**Phenotypic analysis of Patient 0732**

A resistance test vector was constructed as described in example 1 from a patient sample designated as 0732. This

patient had been previously treated with nelfinavir. Isolation of viral RNA and RT/PCR was used to generate a patient derived segment that comprised viral sequences coding for all of PR and aa 1 - 313 of RT. The patient derived segment was inserted into an indicator gene viral vector to generate a resistance test vector designated RTV-0732. RTV-0732 was tested using a phenotypic susceptibility assay to determine accurately and quantitatively the level of susceptibility to a panel of anti-retroviral drugs. This panel of anti-retroviral drugs comprised members of the classes known as NRTIs (AZT, 3TC, d4T, ddI, ddC, and abacavir), NNRTIs (delavirdine, nevirapine and efavirenz), and PRIs (indinavir, nelfinavir, ritonavir, saquinavir and amprenavir). An IC50 was determined for each drug tested. Susceptibility of the patient virus to each drug was examined and compared to known patterns of susceptibility. A pattern of susceptibility to the PRIs was observed for patient sample RTV-0732 in which there was a decrease in both nelfinavir and indinavir susceptibility (increased resistance) and an increase in amprenavir susceptibility (see Fig. 4 and Table 1). Patient sample 0732 was examined further for genotypic changes associated with the pattern of susceptibility.

**Determination of genotype of patient 0732**

RTV-0732 DNA was analyzed by ABI chain terminator automated sequencing. The nucleotide sequence was compared to the consensus sequence of a wild type clade B HIV-1 (HIV Sequence Database Los Alamos, NM). The nucleotide sequence was examined for sequences that are different from the control sequence. PR mutations were

5    noted at positions K14R, I15V, K20T, E35D, M36I, R41K,
     I62V, L63Q and N88S. K14R, I15V, E35D, R41K and I62V are
     naturally occurring polymorphisms in HIV-1 PR and are not
     associated with reduced susceptibility to any drug. M36I
     has previously been described to be associated with
10   resistance to ritonavir and nelfinavir (Shihazi, 1998).
     N88S has previously been described to be associated with
     resistance to nelfinavir (Patick AAC, 42: 2637 (1998) and
     an investigational PRI, SC55389A (Smidt, 1997).


15   **Phenotypic analysis of Patient 627**
     A resistance test vector was constructed as described in
     example 1 from a patient sample designated as 627. This
     patient had been treated with indinavir. Isolation of
     viral RNA and RT/PCR was used to generate a patient
20   derived segment that comprised viral sequences coding for
     all of PR and aa 1 - 313 of RT. The patient derived
     segment was inserted into an indicator gene viral vector
     to generate a resistance test vector designated RTV-627.
     RTV-627 was tested using a phenotypic susceptibility assay
25   to determine accurately and quantitatively the level of
     susceptibility to a panel of anti-retroviral drugs. This
     panel of anti-retroviral drugs comprised members of the
     classes known as NRTIs (AZT, 3TC, d4T, ddI, ddC, and
     abacavir), NNRTIs (delavirdine, nevirapine and efavirenz),
30   and PRIs (indinavir, nelfinavir, ritonavir, saquinavir and
     amprenavir). An IC50 was determined for each drug tested.
     Susceptibility of the patient virus to each drug was
     examined and compared to known patterns of susceptibility.
     A pattern of susceptibility to the PRIs was observed for
35   patient sample RTV-627 in which there was a decrease in
     indinavir and nelfinavir susceptibility (increased

●  ●

-98-

resistance) and an increase in amprenavir and saquinavir susceptibility. Patient sample 627 was examined further for genotypic changes associated with the pattern of susceptibility.

**Determination of genotype of patient 627**

RTV-627 DNA was analyzed by ABI chain terminator automated sequencing. The nucleotide sequence was compared to the consensus sequence of a wild type clade B HIV-1 (HIV Sequence Database Los Alamos, NM). The nucleotide sequence was examined for sequences that are different from the control sequence. PR mutations were noted at positions 13I/V, E35D, M46L, L63P, I64V, I73V and N88S. I13V, E35D and I64V are naturally occurring polymorphisms in HIV-1 PR and are not associated with reduced susceptibility to any drug. M46L has previously been described to be associated with resistance to indinavir and amprenavir. L63P has previously been described to be associated with resistance to indinavir and nelfinavir. N88S has previously been described to be associated with resistance to nelfinavir (Patick, 1998) and an investigational PRI, SC55389A (Smidt, 1997).

**Phenotypic analysis of Patient 1208**

A resistance test vector was constructed as described in example 1 from a patient sample designated as 1208. This patient had been previously treated with nelfinavir. Isolation of viral RNA and RT/PCR was used to generate a patient derived segment that comprised viral sequences coding for all of PR and aa 1 – 313 of RT. The patient derived segment was inserted into an indicator gene viral vector to generate a resistance test vector designated

5     RTV-1208.    RTV-1208 was tested using a phenotypic susceptibility assay to determine accurately and quantitatively the level of susceptibility to a panel of anti-retroviral drugs. This panel of anti-retroviral drugs comprised members of the classes known as NRTIs (AZT, 3TC,

10     d4T, ddI, ddC, and abacavir), NNRTIs (delavirdine, nevirapine and efavirenz), and PRIs (indinavir, nelfinavir, ritonavir, saquinavir and amprenavir). An IC50 was determined for each drug tested. Susceptibility of the patient virus to each drug was examined and compared

15     to known patterns of susceptibility. A pattern of susceptibility to the PRIs was observed for patient sample RTV-1208 in which there was a decrease in indinavir and nelfinavir susceptibility (increased resistance) and an increase in amprenavir susceptibility. Patient sample

20     1208 was examined further for genotypic changes associated with the pattern of susceptibility.

**Determination of genotype of patient 1208**

    RTV-1208 DNA was analyzed by ABI chain terminator

25     automated sequencing. The nucleotide sequence was compared to the consensus sequence of a wild type clade B HIV-1 (HIV Sequence Database Los Alamos, NM). The nucleotide sequence was examined for sequences that are different from the control sequence. PR mutations were

30     noted at positions I62V, L63P, V77I, and N88S. I62V is a naturally occurring polymorphism in HIV-1 PR and is not associated with reduced susceptibility to any drug. L63P has previously been described to be associated with resistance to indinavir and nelfinavir. V77I has

35     previously been described to be associated with resistance to nelfinavir. N88S has previously been described to be

associated with resistance to nelfinavir (Patick, 1998) and an investigational PRI, SC55389A (Smidt, 1997).

**Phenotypic analysis of Patient 360**

A resistance test vector was constructed as described in example 1 from a patient sample designated as 360. This patient had been previously treated with indinavir. Isolation of viral RNA and RT/PCR was used to generate a patient derived segment that comprised viral sequences coding for all of PR and aa 1 - 313 of RT. The patient derived segment was inserted into an indicator gene viral vector to generate a resistance test vector designated RTV-360. RTV-360 was tested using a phenotypic susceptibility assay to determine accurately and quantitatively the level of susceptibility to a panel of anti-retroviral drugs. This panel of anti-retroviral drugs comprised members of the classes known as NRTIs (AZT, 3TC, d4T, ddI, ddC, and abacavir), NNRTIs (delavirdine, nevirapine and efavirenz), and PRIs (indinavir, nelfinavir, ritonavir, saquinavir and amprenavir). An IC50 was determined for each drug tested. Susceptibility of the patient virus to each drug was examined and compared to known patterns of susceptibility. A pattern of susceptibility to the PRIs was observed for patient sample RTV-360 in which there was a decrease in indinavir and nelfinavir susceptibility (increased resistance) and an increase in amprenavir susceptibility. Patient sample 360 was examined further for genotypic changes associated with the pattern of susceptibility.

**Determination of genotype of patient 360**

RTV-360 DNA was analyzed by ABI chain terminator automated sequencing. The nucleotide sequence was compared to the consensus sequence of a wild type clade B HIV-1 (HIV Sequence Database Los Alamos, NM). The nucleotide sequence was examined for sequences that are different from the control sequence. PR mutations were noted at positions I13V, K20M, M36V, N37A, M46I, I62V, L63P, N88S, and I93L. I13V, N37A and I62V are naturally occurring polymorphisms in HIV-1 PR and are not associated with reduced susceptibility to any drug. K20M has previously been described to be associated with resistance to indinavir. M46I has previously been described to be associated with resistance to indinavir, ritonavir, nelfinavir and amprenavir. L63P has previously been described to be associated with resistance to indinavir and nelfinavir. N88S has previously been described to be associated with resistance to nelfinavir (Patick, 1998) and an investigational PRI, SC55389A (Smidt, 1997).

**Phenotypic analysis of Patient 0910**

A resistance test vector was constructed as described in example 1 from a patient sample designated as 0910. This patient had been previously treated with nelfinavir. Isolation of viral RNA and RT/PCR was used to generate a patient derived segment that comprised viral sequences coding for all of PR and aa 1 - 313 of RT. The patient derived segment was inserted into an indicator gene viral vector to generate a resistance test vector designated RTV-0910. RTV-0910 was tested using a phenotypic susceptibility assay to determine accurately and quantitatively the level of susceptibility to a panel of

-102-

anti-retroviral drugs. This panel of anti-retroviral drugs comprised members of the classes known as NRTIs (AZT, 3TC, d4T, ddI, ddC, and abacavir), NNRTIs (delavirdine, nevirapine and efavirenz), and PRIs (indinavir, nelfinavir, ritonavir, saquinavir and amprenavir). An IC50 was determined for each drug tested. Susceptibility of the patient virus to each drug was examined and compared to known patterns of susceptibility. A pattern of susceptibility to the PRIs was observed for patient sample RTV-0910 in which there was a decrease in indinavir and nelfinavir susceptibility (increased resistance) and an increase in amprenavir susceptibility. Patient sample 0910 was examined further for genotypic changes associated with the pattern of susceptibility.

**Determination of genotype of patient 0910**

RTV-0910 DNA was analyzed by ABI chain terminator automated sequencing. The nucleotide sequence was compared to the consensus sequence of a wild type clade B HIV-1 (HIV Sequence Database Los Alamos, NM). The nucleotide sequence was examined for sequences that are different from the control sequence. PR mutations were noted at positions M46I, L63P, V77I, N88S and I93I/L. I13V, K14R, N37D and I193L are naturally occuring polymorphism in HIV-1 PR and is not associated with reduced susceptibility to any drug. V77I has previously been described to be associated with resistance to nelfinavir. M46I has previously been described to be associated with resistance to indinavir, ritonavir, nelfinavir and amprenavir. L63P has previously been described to be associated with resistance to indinavir and nelfinavir. N88S has previously been described to be

5      associated with resistance to nelfinavir (Patick, 1998)
       and an investigational PRI, SC55389A (Smidt, 1997).


       **Phenotypic analysis of Patient 3542**
       A resistance test vector was constructed as described in
10     example 1 from a patient sample designated as 3542.  This
       patient had been treated with indinavir.  Isolation of
       viral RNA and RT/PCR was used to generate a patient
       derived segment that comprised viral sequences coding for
       all of PR and aa 1 - 313 of RT.  The patient derived
15     segment was inserted into an indicator gene viral vector
       to generate a resistance test vector designated RTV-3542.
       RTV-3542 was tested using a phenotypic susceptibility
       assay to determine accurately and quantitatively the level
       of susceptibility to a panel of anti-retroviral drugs.
20     This panel of anti-retroviral drugs comprised members of
       the classes known as NRTIs (AZT, 3TC, d4T, ddI, ddC, and
       abacavir), NNRTIs (delavirdine, nevirapine and efavirenz),
       and PRIs (indinavir, nelfinavir, ritonavir, saquinavir and
       amprenavir). An IC50 was determined for each drug tested.
25     Susceptibility of the patient virus to each drug was
       examined and compared to known patterns of susceptibility.
       A pattern of susceptibility to the PRIs was observed for
       patient sample RTV-3542 in which there was a decrease in
       indinavir,  nelfinavir  and  ritonavir  susceptibility
30     (increased resistance) and an increase in amprenavir
       susceptibility.  Patient sample 3542 was examined further
       for genotypic changes associated with the pattern of
       susceptibility.


35     Determination of genotype of patient 3542
       RTV-3542  DNA  was  analyzed  by  ABI  chain  terminator

automated sequencing. The nucleotide sequence was compared to the consensus sequence of a wild type clade B HIV-1 (HIV Sequence Database Los Alamos, NM). The nucleotide sequence was examined for sequences that are different from the control sequence. PR mutations were noted at positions I13V, K14R, N37D, M46I, L63P, N88S and I93L. K14R and N37A/D are naturally occurring polymorphisms in HIV-1 PR and are not associated with reduced susceptibility to any drug. M46I has previously been described to be associated with resistance to indinavir, ritonavir, nelfinavir and amprenavir. L63P has previously been described to be associated with resistance to indinavir and nelfinavir. N88S has previously been described to be associated with resistance to nelfinavir (Patick, 1998) and an investigational PRI, SC55389A (Smidt, 1997).

**Phenotypic analysis of Patient 3654**

A resistance test vector was constructed as described in example 1 from a patient sample designated as 3654. This patient had been previously treated with ritonavir. Isolation of viral RNA and RT/PCR was used to generate a patient derived segment that comprised viral sequences coding for all of PR and aa 1 - 313 of RT. The patient derived segment was inserted into an indicator gene viral vector to generate a resistance test vector designated RTV-3654. RTV-3654 was tested using a phenotypic susceptibility assay to determine accurately and quantitatively the level of susceptibility to a panel of anti-retroviral drugs. This panel of anti-retroviral drugs comprised members of the classes known as NRTIs (AZT, 3TC, d4T, ddI, ddC, and abacavir), NNRTIs (delavirdine,

5   nevirapine and efavirenz), and PRIs (indinavir,
nelfinavir, ritonavir, saquinavir and amprenavir). An IC50
was determined for each drug tested. Susceptibility of
the patient virus to each drug was examined and compared
to known patterns of susceptibility. A pattern of
10  susceptibility to the PRIs was observed for patient sample
RTV-3654 in which there was a decrease in indinavir and
nelfinavir susceptibility (increased resistance) and an
increase in amprenavir susceptibility. Patient sample
3654 was examined further for genotypic changes associated
15  with the pattern of susceptibility.


**Determination of genotype of patient 3654**

RTV-3654 DNA was analyzed by ABI chain terminator
automated sequencing. The nucleotide sequence was
20  compared to the consensus sequence of a wild type clade B
HIV-1 (HIV Sequence Database Los Alamos, NM). The
nucleotide sequence was examined for sequences that are
different from the control sequence. PR mutations were
noted at positions I13V, R41K, M46I, L63P, V77I, N88S and
25  I93L. I13V, R41K and I93L are naturally occurring
polymorphism in HIV-1 PR and is not associated with
reduced susceptibility to any drug. M46I has previously
been described to be associated with resistance to
indinavir, ritonavir, nelfinavir and amprenavir. L63P has
30  previously been described to be associated with resistance
to indinavir and nelfinavir. V77I has previously been
described to be associated with resistance to nelfinavir.
N88S has previously been described to be associated with
resistance to an investigational PRI, SC55389A (Smidt,
35  1997).

## EXAMPLE 4

### Using Site Directed Mutants To Correlate Genotypes And Phenotypes Associated With Changes in PRI Drug Susceptibility in HIV.

**Site directed mutagenesis**

Resistance test vectors were constructed containing the N88S mutation alone and in combination with other substitutions in PR (L63P, V77I and M46L) known to modulate the HIV susceptibility to PRIs. Mutations were introduced into the resistance test vector using the mega-primer PCR method for site-directed mutagenesis. (Sakar G and Sommar SS (1994) Biotechniques 8(4), 404-407). First, a resistance test vector was constructed that harbors a unique RsrII restriction site 590 bp downstream of the ApaI restriction site. The 590 bp ApaI - RsrII fragment thus contains the entire protease region. This site was introduced by site-specific oligonucleotide-directed mutagenesis using primer #4. All subsequent mutants were constructed by fragment-exchange of the wild-type ApaI - RsrII fragment in the parent vector with the equivalent fragment carrying the respective mutations.

A resistance test vector containing the N88S mutation (N88S-RTV) was tested using the phenotypic susceptibility assay described above and the results were compared to that of a genetically defined resistance test vector that was wild type at position 88. The pattern of phenotypic susceptibility to the PRIs in the N88S-RTV was altered as compared to wild type. In the context of an otherwise wild type background (i.e. N88S mutation alone) the N88S-RTV was more susceptible to both amprenavir and

ritonavir and slightly less susceptible to nelfinavir compared to the wild type control RTV (see Table 2).

A resistance test vector containing the N88S mutation along with the L63P mutation (L63P-N88S-RTV) was tested using the phenotypic susceptibility assay described above and the results were compared to that of a genetically defined resistance test vector that was wild type at positions 63 and 88. The L63P-N88S-RTV showed decreased susceptibility to both indinavir and nelfinavir and an increase in the susceptibility to amprenavir compared the wild-type control RTV (see Table 2). Thus it appears that the introduction of a second mutation, L63P, in addition to N88S, results in a reduction in susceptibility to nelfinavir and indinavir while the increased susceptibility to amprenavir is maintained.

A resistance test vector containing the N88S mutation along with the L63P mutation and the V77I mutation (L63P-V77I-N88S-RTV) was tested using the phenotypic susceptibility assay described above and the results were compared to that of a genetically defined resistance test vector that was wild type at positions 63 and 77 and 88. The RTV containing mutations at these positions, L63P-V77I-N88S-RTV, showed a decrease in susceptibility to both indinavir and nelfinavir and an increase in the susceptibility to amprenavir compared to the wild-type control RTV (see Fig. 5 and Table 2). Thus it appears that the introduction of a third mutation, V77I, in addition to L63P and N88S, results in a reduction in susceptibility to nelfinavir and indinavir while the increased susceptibility to amprenavir is maintained.

5      The N88S mutation was also introduced into an RTV
       containing additional mutations at positions L63P and M46L
       (M46L + L63P + N88S). The RTV containing mutations at
       these positions, M46L-L63P-N88S-RTV showed a decrease in
10     susceptibility to nelfinavir and a slight decrease in
       susceptibility to indinavir and an increase in the
       susceptibility to amprenavir compared to the wild-type
       control RTV (see Fig. 5 and Table 2). Thus it appears
       that the introduction of a third mutation, M46L, in
15     addition to L63P and N88S, results in a reduction in
       susceptibility to nelfinavir and indinavir while the
       increased susceptibility to amprenavir is maintained.

       A resistance test vector containing the N88S mutation
20     along with the M46L mutation, the L63P mutation, and the
       V77I mutation (M46L-L63P-V77I-N88S-RTV) was tested using
       the phenotypic susceptibility assay described above and
       the results were compared to that of a genetically defined
       resistance test vector that was wild type at positions 46,
25     63, 77 and 88. The RTV containing mutations at these four
       positions, M46L-L63P-V77I-N88S-RTV showed a decrease in
       susceptibility to nelfinavir and indinavir and an increase
       in the susceptibility to amprenavir compared to the
       wild-type control RTV (see Fig. 5 and Table 2). Thus it
30     appears that the introduction of a fourth mutation, V77I,
       in addition to L63P, M46L and N88S results in a reduction
       in susceptibility to nelfinavir and indinavir while the
       increased susceptibility to amprenavir is maintained.
       A resistance test vector containing the L63P mutation
35     (L63P-RTV) was tested using the phenotypic susceptibility
       assay described above and the results were compared to

that of a genetically defined resistance test vector that was wild type at position 63.  The pattern of phenotypic susceptibility to the PRIs in the L63P-RTV was similar to wild type with no significant changes in susceptibility to the PRIs observed.

The L63P mutation was also introduced into an RTV containing an additional mutation at position V77I.  The L63P-V77I-RTV showed a slight decrease in susceptibility to nelfinavir compared to the wild-type control RTV (see Fig. 5 and Table 2).

## EXAMPLE 5

### Predicting Response to Protease Inhibitors by
### Characterization of Amino Acid 88 of HIV-1 Protease.

In one embodiment of this invention, changes in the amino acid at position 88 of the protease protein of HIV-1 is evaluated using the following method comprising: (i) collecting a biological sample from an HIV-1 infected subject; (ii) evaluating whether the biological sample contains nucleic acid encoding HIV-1 protease having an asparagine to serine mutation at codon 88 (N88S); and (iii) determining susceptibility to protease inhibitors (PRI).

The biological sample comprises whole blood, blood components including peripheral mononuclear cells (PBMC), serum, plasma (prepared using various anticoagulants such as EDTA, acid citrate-dextrose, heparin), tissue biopsies, cerebral spinal fluid (CSF), or other cell, tissue or body fluids.  In another embodiment, the HIV-1 nucleic acid

5      (genomic RNA) or reverse transcriptase protein can be
       isolated directly from the biological sample or after
       purification of virus particles from the biological
       sample. Evaluating whether the amino acid at position 88
       of the HIV-1 protease is mutated to serine, can be
10     performed using various methods, such as direct
       characterization of the viral nucleic acid encoding
       protease or direct characterization of the protease
       protein itself. Defining the amino acid at position 88 of
       protease can be performed by direct characterization of
15     the protease protein by conventional or novel amino acid
       sequencing methodologies, epitope recognition by
       antibodies or other specific binding proteins or
       compounds. Alternatively, the amino acid at position 88
       of the HIV-1 protease protein can be defined by
20     characterizing amplified copies of HIV-1 nucleic acid
       encoding the protease protein. Amplification of the HIV-1
       nucleic acid can be performed using a variety of
       methodologies including reverse transcription-polymerase
       chain reaction (RT-PCR), NASBA, SDA, RCR, or 3SR. The
25     nucleic acid sequence encoding HIV protease at codon 88
       can be determined by direct nucleic acid sequencing using
       various primer extension-chain termination (Sanger, ABI/PE
       and Visible Genetics) or chain cleavage (Maxam and
       Gilbert) methodologies or more recently developed
30     sequencing methods such as matrix assisted laser
       desorption-ionization time of flight (MALDI-TOF) or mass
       spectrometry (Sequenom, Gene Trace Systems).
       Alternatively, the nucleic acid sequence encoding amino
       acid position 88 can be evaluated using a variety of probe
35     hybridization methodologies, such as genechip
       hybridization sequencing (Affymetrix), line probe assay

5      (LiPA; Murex), and differential hybridization (Chiron).

       In a preferred embodiment of this invention, evaluation of
       protease inhibitor susceptibility and of whether amino
       acid position 88 of HIV-1 protease was wild type or serine
10     was carried out using a phenotypic susceptibility assay or
       genotypic assay, respectively, using resistance test
       vector DNA prepared from the biological sample. In one
       embodiment, the plasma sample was collected, viral RNA was
       purified and an RT-PCR methodology was used to amplify a
15     patient derived segment encoding the HIV-1 protease and
       reverse transcriptase regions. The amplified patient
       derived segments were then incorporated, via DNA ligation
       and bacterial transformation, into an indicator gene viral
       vector thereby generating a resistance test vector.
20     Resistance test vector DNA was isolated from the bacterial
       culture and the phenotypic susceptibility assay was
       carried out as described in Example 1. The results of the
       phenotypic susceptibility assay with a patient sample
       having an N88S mutation in PR is shown in Figure 4. The
25     nucleic acid (DNA) sequence of the patient derived HIV-1
       protease and reverse transcriptase regions from patient
       sample 0732 was determined using a fluorescence detection
       chain termination cycle sequencing methodology (ABI/PE).
       The method was used to determine a consensus nucleic acid
30     sequence representing the combination of sequences of the
       mixture of HIV-1 variants existing in the subject sample
       (representing the quasispecies), and to determine the
       nucleic acid sequences of individual variants.

35     **Phenotypic and genotypic correlation of mutations at amino
       acid 88 of HIV-1 Protease.**

Phenotypic susceptibility profiles of patient samples and site directed mutants showed that amprenavir susceptibility correlated with the presence of the N88S mutation in HIV-1 protease. Phenotypic susceptibility profiles of patient samples and site directed mutants showed that a significant increase in amprenavir susceptibility (decreased resistance) correlated with a mutation in the nucleic acid sequence encoding the amino acid serine (S) at position 88 of HIV-1 protease.

Phenotypic susceptibility profiles of patient samples and site directed mutants showed reduction in amprenavir susceptibility (decreased resistance) and a decrease in susceptibility to nelfinavir and indinavir with the amino acid serine at position 88 when the PR mutations at positions 63, 77 or 46 were also present (L63P, V77I, or M46L).

## EXAMPLE 6

### Using Resistance test vectors and site directed mutants to correlate genotypes associated with alterations in PRI susceptibility with viral fitness.

Luciferase activity measured in the absence of drug for the seven resistance test vectors constructed from the patient viruses containing the N88S PR mutation ranged from 0.7 to 16% of control (Table 3). Although these viruses also contain multiple mutations in reverse transcriptase, which could also contribute to a reduction in viral fitness, the data suggest that viruses containing the N88S mutation are less fit than wild type. To confirm

this observation, the luciferase expression level for the
site-directed mutant resistance test vectors was also
examined.

Viruses containing N88S as the only substitution produced
only 1.0% of the luciferase activity in the absence of
drug (Table 4). This reduction was substantially
alleviated by the addition of the L63P substitution
(20.7%) or by addition of the combinations of L63P/V77I
(29.3%) or M46L/L63P (28.0%). The L63P or L63P/V77I
mutants had equivalent or increased relative luciferase
activity compared to wild type (163.9 and 75.6%,
respectively).

When the K20T substitution was added to the N88S
background, either alone or in combination with L63P, only
background levels of luciferase activity was detected.
Sequence analysis confirmed the absence of additional
mutations, which might render the vector inactive. Thus
the combination of the K20T and N88S substitutions
correlates with a severe defect in fitness.

## EXAMPLE 7

### Predicting Response to Protease Inhibitors by
### Characterization of Amino Acid 82 of HIV-1
### Protease.

In one embodiment of this invention, changes in the amino
acid at position 82 of the protease protein of HIV-1 are
evaluated using the following method comprising: (i)
collecting a biological sample from an HIV-1 infected
subject; (ii) evaluating whether the biological sample

contains nucleic acid encoding HIV-1 protease having a valine to alanine (V82A), phenylalanine (V82F), serine (V82S), or threonine (V82T) substitution at codon 82; and (iii) determining susceptibility to protease inhibitors (PRI).

The biological sample comprises whole blood, blood components including peripheral mononuclear cells (PBMC), serum, plasma (prepared using various anticoagulants such as EDTA, acid citrate-dextrose, heparin), tissue biopsies, cerebral spinal fluid (CSF), or other cell, tissue or body fluids. In another embodiment, the HIV-1 nucleic acid (genomic RNA) or reverse transcriptase protein can be isolated directly from the biological sample or after purification of virus particles from the biological sample. Evaluating whether the amino acid at position 82 of the HIV-1 protease is mutated to alanine, phenylalanine, serine, or threonine, can be performed using various methods, such as direct characterization of the viral nucleic acid encoding protease or direct characterization of the protease protein itself. Defining the amino acid at position 82 of protease can be performed by direct characterization of the protease protein by conventional or novel amino acid sequencing methodologies, epitope recognition by antibodies or other specific binding proteins or compounds. Alternatively, the amino acid at position 82 of the HIV-1 protease protein can be defined by characterizing amplified copies of HIV-1 nucleic acid encoding the protease protein. Amplification of the HIV-1 nucleic acid can be performed using a variety of methodologies including reverse transcription-polymerase chain reaction (RT-PCR), NASBA,

5      SDA, RCR, or 3SR. The nucleic acid sequence encoding HIV protease at codon 82 can be determined by direct nucleic acid sequencing using various primer extension-chain termination (Sanger, ABI/PE and Visible Genetics) or chain cleavage (Maxam and Gilbert) methodologies or more

10      recently developed sequencing methods such as matrix assisted laser desorption-ionization time of flight (MALDI-TOF) or mass spectrometry (Sequenom, Gene Trace Systems). Alternatively, the nucleic acid sequence encoding amino acid position 82 can be evaluated using a

15      variety of probe hybridization methodologies, such as genechip hybridization sequencing (Affymetrix), line probe assay (LiPA, Murex), and differential hybridization (Chiron).

20      In a preferred embodiment of this invention, evaluation of protease inhibitor susceptibility and of whether amino acid position 82 of HIV-1 protease was wild type or alanine, phenylalanine, serine, or threonine, was carried out using a phenotypic susceptibility assay or genotypic

25      assay, respectively, using resistance test vector DNA prepared from the biological sample. In one embodiment, the plasma sample was collected, viral RNA was purified and an RT-PCR methodology was used to amplify a patient derived segment encoding the HIV-1 protease and reverse

30      transcriptase regions. The amplified patient derived segments were then incorporated, via DNA ligation and bacterial transformation, into an indicator gene viral vector thereby generating a resistance test vector. Resistance test vector DNA was isolated from the bacterial

35      culture and the phenotypic susceptibility assay was carried out and analyzed as described in Example 1.

5      The nucleic acid (DNA) sequence of the patient derived
       HIV-1 protease and reverse transcriptase regions was
       determined using a fluorescence detection chain
       termination cycle sequencing methodology (ABI/PE). The
10     method was used to determine a consensus nucleic acid
       sequence representing the combination of sequences of the
       mixture of HIV-1 variants existing in the subject sample
       (representing the quasispecies), and to determine the
       nucleic acid sequences of individual variants. Genotypes
15     are analyzed as lists of amino acid differences between
       virus in the patient sample and a reference laboratory
       strain of HIV-1, NL4-3. Genotypes and corresponding
       phenotypes (fold-change in IC50 values) are entered in a
       relational database linking these two results with patient
20     information. Large datasets can then be assembled from
       patient virus samples sharing particular characteristics,
       such as the presence of any given mutation, or combination
       of mutations or reduced susceptibility to any drug or
       combination of drugs.

25

       (a)   Protease   inhibitor   susceptibility   of   viruses
       containing mutations at amino acid 82 of HIV-1 Protease.

       Phenotypic susceptibility profiles of 75 patient virus
30     samples which contained a mutation at position 82 (V82A,
       F, S, or T), but no other primary mutations, were
       analyzed. According to most published guidelines, such
       viruses are expected to be resistant to ritonavir,
       nelfinavir, indinavir, and saquinavir. However, 8%, 20%,
35     23%, and 73% of these samples were phenotypically
       susceptible to these four protease inhibitors,

-117-

respectively (see Table 6).    Thus, particularly for indinavir and saquinavir, there was poor correlation between the presence of mutations at position 82 and drug susceptibility.

**(b)  Indinavir  susceptibility  of  viruses  containing combinations  of  mutations  at  amino  acid  82  and  one secondary mutation in HIV-1 Protease.**

Indinavir resistance in viruses containing mutations at position 82 was evaluated with respect to the presence of other  specific  mutations.    Decreased  indinavir susceptibility (fold-change in $IC_{50}$ greater than 2.5) in viruses containing V82A, F, S, or T but no other primary mutations was correlated with the presence of mutations at secondary positions.  Reduced indinavir susceptibility was observed  in  20  samples  containing  mutations  at  both positions 24 and 82 (100%) and in 27 samples with both 71 and 82 (100%) (See Table 7). The combination of mutations at position 82 with mutations at other positions (e.g. 54, 46,  10,  and  63)  also  significantly  increased  the proportion  of  samples  that  had  reduced  indinavir susceptibility (Table 7).

**(c)  Saquinavir  susceptibility  of  viruses  containing combinations  of  mutations  at  amino  acid  82  and  one secondary mutation in HIV-1 Protease.**

Saquinavir resistance in viruses containing mutations at position 82 was evaluated with respect to the presence of other  specific  mutations.    Decreased  saquinavir susceptibility (fold-change in $IC_{50}$ greater than 2.5) in viruses containing V82A, F, S, or T but no other primary mutations was correlated with the presence of mutations at

secondary positions. Reduced saquinavir susceptibility was observed in 4 of 5 samples containing mutations at both positions 20 and 82 (80%) and in 8 of 11 samples with both 36 and 82 (73%) (See Table 8). The combination of mutations at position 82 with mutations at other positions (e.g. 24, 71, 54, and 10) also significantly increased the proportion of samples that had reduced saquinavir susceptibility (Table 8).

**(d)   Indinavir   susceptibility   of   viruses   containing combinations   of   mutations   at   amino   acid   82   and   many secondary mutations in HIV-1 Protease.**

Indinavir resistance in viruses containing mutations at position 82 was evaluated with respect to the presence of a defined number of other mutations. Decreased indinavir susceptibility (fold-change in $IC_{5c}$ greater than 2.5) in viruses containing V82A, F, S, or T but no other primary mutations was correlated with the number of mutations at secondary positions. Reduced indinavir susceptibility was observed in 100% of samples with V82A, F, S, or T and at least 6 other secondary mutations (See Table 9). The proportion of samples that had reduced indinavir susceptibility increased significantly in samples with V82A, F, S, or T combined with 3 to 5 other secondary mutations (Table 9).

**(e)   Saquinavir   susceptibility   of   viruses   containing combinations   of   mutations   at   amino   acid   82   and   many secondary mutations in HIV-1 Protease.**

Saquinavir resistance in viruses containing mutations at position 82 was evaluated with respect to the presence of a defined number of other mutations. Decreased saquinavir

5  susceptibility (fold-change in $IC_{50}$ greater than 2.5) in
   viruses containing V82A, F, S, or T but no other primary
   mutations was correlated with the number of mutations at
   secondary positions.   Reduced saquinavir susceptibility
   was observed in 60 to 76% of samples with V82A, F, S, or T
10 and at least 5 other secondary mutations (See Table 9).
   The proportion of samples that had reduced saquinivir
   susceptibility increased significantly in samples with
   V82A, F, S, or T combined with 3 or 4 other secondary
   mutations (Table 9).

15

**EXAMPLE 8**

**Predicting Response to Protease Inhibitors by**
**Characterization of Amino Acid 90 of HIV-1**
**Protease.**

20

   In one embodiment of this invention, changes in the amino
   acid at position 90 of the protease protein of HIV-1 are
   evaluated using the following method comprising: (i)
   collecting a biological sample from an HIV-1 infected
25 subject; (ii) evaluating whether the biological sample
   contains nucleic acid encoding HIV-1 protease having a
   leucine to methionine (L90M) substitution at codon 90; and
   (iii) determining susceptibility to protease inhibitors
   (PRI).

30

   The biological sample comprises whole blood, blood
   components including peripheral mononuclear cells (PBMC),
   serum, plasma (prepared using various anticoagulants such
   as EDTA, acid citrate-dextrose, heparin), tissue biopsies,
35 cerebral spinal fluid (CSF), or other cell, tissue or body
   fluids.   In another embodiment, the HIV-1 nucleic acid

(genomic RNA) or reverse transcriptase protein can be isolated directly from the biological sample or after purification of virus particles from the biological sample. Evaluating whether the amino acid at position 90 of the HIV-1 protease is mutated to methionine, can be performed using various methods, such as direct characterization of the viral nucleic acid encoding protease or direct characterization of the protease protein itself. Defining the amino acid at position 90 of protease can be performed by direct characterization of the protease protein by conventional or novel amino acid sequencing methodologies, epitope recognition by antibodies or other specific binding proteins or compounds. Alternatively, the amino acid at position 90 of the HIV-1 protease protein can be defined by characterizing amplified copies of HIV-1 nucleic acid encoding the protease protein. Amplification of the HIV-1 nucleic acid can be performed using a variety of methodologies including reverse transcription-polymerase chain reaction (RT-PCR), NASBA, SDA, RCR, or 3SR. The nucleic acid sequence encoding HIV protease at codon 90 can be determined by direct nucleic acid sequencing using various primer extension-chain termination (Sanger, ABI/PE and Visible Genetics) or chain cleavage (Maxam and Gilbert) methodologies or more recently developed sequencing methods such as matrix assisted laser desorption-ionization time of flight (MALDI-TOF) or mass spectrometry (Sequenom, Gene Trace Systems). Alternatively, the nucleic acid sequence encoding amino acid position 90 can be evaluated using a variety of probe hybridization methodologies, such as genechip hybridization sequencing (Affymetrix), line probe assay

(LiPA; Murex), and differential hybridization (Chiron).

In a preferred embodiment of this invention, evaluation of protease inhibitor susceptibility and of whether amino acid position 90 of HIV-1 protease was wild type or methionine, was carried out using a phenotypic susceptibility assay or genotypic assay, respectively, using resistance test vector DNA prepared from the biological sample. In one embodiment, the plasma sample was collected, viral RNA was purified and an RT-PCR methodology was used to amplify a patient derived segment encoding the HIV-1 protease and reverse transcriptase regions. The amplified patient derived segments were then incorporated, via DNA ligation and bacterial transformation, into an indicator gene viral vector thereby generating a resistance test vector. Resistance test vector DNA was isolated from the bacterial culture and the phenotypic susceptibility assay was carried out and analyzed as described in Example 1.

The nucleic acid (DNA) sequence of the patient derived HIV-1 protease and reverse transcriptase regions was determined using a fluorescence detection chain termination cycle sequencing methodology (ABI/PE). The method was used to determine a consensus nucleic acid sequence representing the combination of sequences of the mixture of HIV-1 variants existing in the subject sample (representing the quasispecies), and to determine the nucleic acid sequences of individual variants. Genotypes are analyzed as lists of amino acid differences between virus in the patient sample and a reference laboratory strain of HIV-1, NL4-3. Genotypes and corresponding

phenotypes (fold-change in IC50 values) are entered in a relational database linking these two results with patient information. Large datasets can then be assembled from patient virus samples sharing particular characteristics, such as the presence of any given mutation, or combination of mutants, or reduced susceptibility to any drug or combination of drugs.

**(a) Protease inhibitor susceptibility of viruses containing mutations at amino acid 90 of HIV-1 Protease.**

Phenotypic susceptibility profiles of 58 patient virus samples which contained a mutation at position 90 (L90M), but no other primary mutations, were analyzed. According to most published guidelines, such viruses are expected to be resistant to ritonavir, nelfinavir, indinavir, and saquinavir. However, 28%, 9%, 31%, and 47% of these samples were phenotypically susceptible to these four protease inhibitors, respectively (see Table 6). Thus, particularly for indinavir and saquinavir, there was poor correlation between the presence of mutations at position 90 and drug susceptibility.

**(b) Indinavir susceptibility of viruses containing combinations of mutations at amino acid 90 and one secondary mutation in HIV-1 Protease.**

Indinavir resistance in viruses containing mutations at position 90 was evaluated with respect to the presence of other specific mutations. Decreased indinavir susceptibility (fold-change in $IC_{50}$ greater than 2.5) in viruses containing L90M but no other primary mutations was correlated with the presence of mutations at secondary

5      positions.  Reduced indinavir susceptibility was observed
       in 17 of 19 samples containing mutations at both positions
       73 and 90 (89%) and in 16 of 18 samples with both 71 and
       90 (89%) (See Table 10).  The combination of mutations at
       position    90    with    mutation    at    position    46    also
10     significantly increased the proportion of samples that had
       reduced indinavir susceptibility (Table 10).


       *(c)  Saquinavir  susceptibility  of  viruses  containing
       combinations  of  mutations  at  amino  acid  90  and  one
15     secondary mutation in HIV-1 Protease.*
       Saquinavir resistance in viruses containing mutations at
       position 90 was evaluated with respect to the presence of
       other    specific    mutations.         Decreased    saquinavir
       susceptibility (fold-change in $IC_{50}$ greater than 2.5) in
20     viruses containing L90M but no other primary mutations was
       correlated with the presence of mutations at secondary
       positions.  Reduced saquinavir susceptibility was observed
       in 15 of 19 samples containing mutations at both positions
       73 and 90 (79%) and in 14 of 18 samples with both 71 and
25     90 (78%) (See Table 11).  The combination of mutations at
       position 90 with mutations at other positions (e.g. 77 and
       10) also significantly increased the proportion of samples
       that had reduced saquinavir susceptibility (Table 1).


30


       *(d)   Indinavir  susceptibility  of  viruses  containing
       combinations  of  mutations  at  amino  acid  90  and  many
35     secondary mutations in HIV-1 Protease.*
       Indinavir resistance in viruses containing mutations at

5      position 90 was evaluated with respect to the presence of
       a defined number of other mutations. Decreased indinavir
       susceptibility (fold-change in $IC_{50}$ greater than 2.5) in
       viruses containing L90M but no other primary mutations was
       correlated with the number of mutations at secondary
10     positions. Reduced indinavir susceptibility was observed
       in 100% of samples with L90M and at least 5 other
       secondary mutations had (See Table 12). The proportion of
       samples that had reduced indinavir susceptibility
       increased significantly in samples with L90M combined with
15     3 or 4 other secondary mutations (Table 12).

       *(e) Saquinavir susceptibility of viruses containing
       combinations of mutations at amino acid 90 and many
       secondary mutations in HIV-1 Protease.*

20     Saquinavir resistance in viruses containing mutations at
       position 90 was evaluated with respect to the presence of
       a defined number of other mutations. Decreased saquinavir
       susceptibility (fold-change in $IC_{5c}$ greater than 2.5) in
       viruses containing L90M but no other primary mutations was
25     correlated with the number of mutations at secondary
       positions. Reduced saquinavir susceptibility was observed
       in 100% of samples with L90M and at least 5 other
       secondary mutations (See Table 12). The proportion of
       samples that had reduced saquinivir susceptibility
30     increased significantly in samples with L90M combined with
       3 or 4 other secondary mutations (Table 12).

                              **EXAMPLE 9**
                  **Predicting Response to Protease Inhibitors by**
35           **Characterization of Amino Acids 82 and 90 of HIV-1**
                              **Protease.**

-125-

In one embodiment of this invention, changes in the amino acid at position 82 and 90 of the protease protein of HIV-1 are evaluated using the following method comprising: (i) collecting a biological sample from an HIV-1 infected subject; (ii) evaluating whether the biological sample contains nucleic acid encoding HIV-1 protease having a valine to alanine (V82A), phenylalanine (V82F), serine (V82S), or threonine (V82T) substitution at codon 82 or a leucine to methionine at position 90 (L90M); and (iii) determining susceptibility to protease inhibitors (PRI).

The biological sample comprises whole blood, blood components including peripheral mononuclear cells (PBMC), serum, plasma (prepared using various anticoagulants such as EDTA, acid citrate-dextrose, heparin), tissue biopsies, cerebral spinal fluid (CSF), or other cell, tissue or body fluids.  In another embodiment, the HIV-1 nucleic acid (genomic RNA) or reverse transcriptase protein can be isolated directly from the biological sample or after purification of virus particles from the biological sample. Evaluating whether the amino acid at position 82 of the HIV-1 protease is mutated to alanine, phenylalanine, serine, or threonine or at position 90 to methionine, can be performed using various methods, such as direct characterization of the viral nucleic acid encoding protease or direct characterization of the protease protein itself.  Defining the amino acid at positions 82 and 90 of protease can be performed by direct characterization of the protease protein by conventional or novel amino acid sequencing methodologies, epitope recognition by antibodies or other specific binding

5      proteins or compounds.  Alternatively, the amino acid at
       positions 82 and 90 of the HIV-1 protease protein can be
       defined by characterizing amplified copies of HIV-1
       nucleic acid encoding the protease protein. Amplification
       of the HIV-1 nucleic acid can be performed using a variety
10     of   methodologies   including   reverse
       transcription-polymerase chain reaction (RT-PCR), NASBA,
       SDA, RCR, or 3SR. The nucleic acid sequence encoding HIV
       protease at codons 82 and 90 can be determined by direct
       nucleic   acid   sequencing   using   various   primer
15     extension-chain termination (Sanger, ABI/PE and Visible
       Genetics)   or   chain   cleavage   (Maxam   and   Gilbert)
       methodologies   or   more   recently   developed   sequencing
       methods   such   as   matrix   assisted   laser
       desorption-ionization time of flight (MALDI-TOF) or mass
20     spectrometry   (Sequenom,   Gene   Trace   Systems).
       Alternatively, the nucleic acid sequence encoding amino
       acid positions 82 and 90 can be evaluated using a variety
       of probe hybridization methodologies, such as genechip
       hybridization sequencing (Affymetrix), line probe assay
25     (LiPA; Murex), and differential hybridization (Chiron).

       In a preferred embodiment of this invention, evaluation of
       protease inhibitor susceptibility and of whether' amino
       acid positions 82 and 90 of HIV-1 protease was wild type
30     or alanine, phenylalanine, serine, or threonine in the
       case of position 82 and methionine at position 90, was
       carried out using a phenotypic susceptibility assay or
       genotypic assay, respectively, using resistance test
       vector DNA prepared from the biological sample.  In one
35     embodiment, plasma sample was collected, viral RNA was
       purified and an RT-PCR methodology was used to amplify a

● ●

5    patient derived segment encoding the HIV-1 protease and
     reverse transcriptase regions.  The amplified patient
     derived segments were then incorporated, via DNA ligation
     and bacterial transformation, into an indicator gene viral
     vector thereby generating a resistance test vector.
10   Resistance test vector DNA was isolated from the bacterial
     culture and the phenotypic susceptibility assay was
     carried out and analyzed as described in Example 1.

     The nucleic acid (DNA) sequence of the patient derived
15   HIV-1 protease and reverse transcriptase regions was
     determined using a fluorescence detection chain
     termination cycle sequencing methodology (ABI/PE).  The
     method was used to determine a consensus nucleic acid
     sequence representing the combination of sequences of the
20   mixture of HIV-1 variants existing in the subject sample
     (representing the quasispecies), and to determine the
     nucleic acid sequences of individual variants. Genotypes
     are analyzed as lists of amino acid differences between
     virus in the patient sample and a reference laboratory
25   strain of HIV-1, NL4-3. Genotypes and corresponding
     phenotypes (fold-change in IC50 values) are entered in a
     relational database linking these two results with patient
     information.  Large datasets can then be assembled from
     patient virus samples sharing particular characteristics,
30   such as the presence of any given mutation or reduced
     susceptibility to any drug or combination of drugs.

     **Protease inhibitor susceptibility of viruses containing
     mutations at amino acids 82 and 90 of HIV-1 Protease.**
35   Phenotypic susceptibility profiles of 33 patient virus
     samples which contained mutations at positions 82 (V82A,

5      F, S, or T) and 90 (L90M), but no other primary mutations, were analyzed. According to most published guidelines, such viruses are expected to be resistant to ritonavir, nelfinavir, indinavir, and saquinavir. However, 9% and 21% of these samples were phenotypically susceptible to

10    indinavir and saquinavir, respectively (see Table 6). Thus, particularly for saquinavir, there was poor correlation between the presence of mutations at positions 82 and 90 and drug susceptibility.

15

**EXAMPLE 10**

**Measuring Replication Fitness Using Resistance Test**

20    **Vectors**

A means and method is provided for accurately measuring and reproducing the replication fitness of HIV-1. This method for measuring replication fitness is applicable to

25    other viruses, including, but not limited to hepadnaviruses (human hepatitis B virus), flaviviruses (human hepatitis C virus) and herpesviruses (human cytomegalovirus). This example further provides a means and method for measuring the replication fitness of HIV-1

30    that exhibits reduced drug susceptibility to reverse transcriptase inhibitors and protease inhibitors. This method can be used for measuring replication fitness for other classes of inhibitors of HIV-1 replication, including, but not limited to integration, virus assembly,

35    and virus attachment and entry.

Replication fitness tests are carried out using the means and methods for phenotypic drug susceptibility and resistance tests described in US Patent Number 5,837,464 (International Publication Number WO 97/27319) which is hereby incorporated by reference.

In these experiments patient-derived segment(s) corresponding to the HIV protease and reverse transcriptase coding regions were either patient-derived segments amplified by the reverse transcription-polymerase chain reaction method (RT-PCR) using viral RNA isolated from viral particles present in the serum of HIV-infected individuals or were mutants of wild type HIV-1 made by site directed mutagenesis of a parental clone of resistance test vector DNA. Resistance test vectors are also referred to as "fitness test vectors" when used to evaluate replication fitness. Isolation of viral RNA was performed using standard procedures (e.g. RNAgents Total RNA Isolation System, Promega, Madison WI or RNAzol, Tel-Test, Friendswood, TX). The RT-PCR protocol was divided into two steps. A retroviral reverse transcriptase [e.g. Moloney MuLV reverse transcriptase (Roche Molecular Systems, Inc., Branchburg, NJ), or avian myeloblastosis virus (AMV) reverse transcriptase, (Boehringer Mannheim, Indianapolis, IN)] was used to copy viral RNA into cDNA. The cDNA was then amplified using a thermostable DNA polymerase [e.g. Taq (Roche Molecular Systems, Inc., Branchburg, NJ), Tth (Roche Molecular Systems, Inc., Branchburg, NJ), PrimeZyme (isolated from Thermus brockianus, Biometra, Gottingen, Germany)] or a combination of thermostable polymerases as described for the performance of "long PCR" (Barnes, W.M., (1994) Proc.

5      Natl. Acad. Sci, USA 91, 2216-2220) [e.g. Expand High
       Fidelity PCR System (Taq + Pwo), (Boehringer Mannheim.
       Indianapolis, IN) OR GeneAmp XL PCR kit (Tth + Vent),
       (Roche Molecular Systems, Inc., Branchburg, NJ)].


10     PCR6 (Table 5, #1) is used for reverse transcription of
       viral RNA into cDNA.  The primers, ApaI primer (PDSApa,
       Table 5, #2) and AgeI primer (PDSAge, Table 5, #3) used to
       amplify the "test" patient-derived segments contained
       sequences resulting in ApaI and AgeI recognition sites
15     being introduced into both ends of the PCR product,
       respectively.


       Fitness test vectors incorporating the "test"
20     patient-derived segments were constructed as described in
       US Patent Number 5,837,464 (International Publication
       Number WO 97/27319) (see Fig. 1) using an amplified DNA
       product of 1.5 kB prepared by RT-PCR using viral RNA as a
       template and oligonucleotides PCR6 (#1), PDSApa (#2) and
25     PDSAge (#3) as primers, followed by digestion with ApaI
       and AgeI or the isoschizomer PinAl.  To ensure that the
       plasmid DNA corresponding to the resultant fitness test
       vector comprises a representative sample of the HIV viral
       quasi-species present in the serum of a given patient,
30     many (>100) independent E. coli transformants obtained in
       the construction of a given fitness test vector were
       pooled and used for the preparation of plasmid DNA.


       A packaging expression vector encoding an amphotrophic
35     MuLV 4070A env gene product enables production in a
       fitness test vector host cell of fitness test vector viral

5      particles which can efficiently infect human target cells.
       Fitness test vectors encoding all HIV genes with the
       exception of env were used to transfect a packaging host
       cell (once transfected the host cell is referred to as a
       fitness test vector host cell). The packaging expression
10     vector which encodes the amphotrophic MuLV 4070A env gene
       product is used with the resistance test vector to enable
       production in the fitness test vector host cell of
       infectious   pseudotyped   fitness   test   vector   viral
       particles.
15

       Fitness tests performed with fitness test vectors were
       carried out using packaging host and target host cells
       consisting of the human embryonic kidney cell line 293
       (Cell Culture Facility, UC San Francisco, SF, CA)..
20

       Fitness tests were carried out with fitness test vectors
       using two host cell types. Fitness test vector viral
       particles were produced by a first host cell (the fitness
       test vector host cell) that was prepared by transfecting a
25     packaging host cell with the fitness test vector and the
       packaging expression vector. The fitness test vector
       viral particles were then used to infect a second host
       cell (the target host cell) in which the expression of the
       indicator gene is measured (see Fig. A).
30

       The   fitness   test   vectors   containing   a   functional
       luciferase gene cassette were constructed and host cells
       were transfected with the fitness test vector DNA. The
       fitness test vectors contained patient-derived reverse
35     transcriptase and protease DNA sequences that encode
       proteins which were either susceptible or resistant to the

5    antiretroviral    agents,    such    as    nucleoside    reverse
     transcriptase    inhibitors,    non-nucleoside    reverse
     transcriptase inhibitors and protease inhibitors._

     The amount of luciferase activity detected in the infected
10   cells  is  used  as  a  direct  measure  of  "infectivity",
     "replication capacity" or "fitness", i.e. the ability of
     the  virus  to  complete  a  single  round  of  replication.
     Relative fitness is assessed by comparing the amount of
     luciferase activity produced by patient derived viruses to
15   the  amount  of  luciferase  activity  produced  by  a  well-
     characterized reference virus (wildtype) derived from a
     molecular  clone  of  HIV-1,  for  example  NL4-3  or  HXB2.
     Fitness  measurements  are  expressed  as  a  percent  of  the
     reference,  for  example  25%,  50%,  75%,  100%  or  125%  of
20   reference (Figure B, C).

     Host  cells  were  seeded  in  10-cm-diameter  dishes  and  were
     transfected one day after plating with fitness test vector
     plasmid   DNA   and   the   envelope   expression   vector.
25   Transfections  were  performed  using  a  calcium-phosphate
     co-precipitation  procedure.    The   cell   culture   media
     containing the DNA precipitate was replaced with fresh
     medium,  from  one  to  24  hours,  after  transfection.   Cell
     culture  media  containing  fitness  test  vector  viral
30   particles  was  harvested  one  to  four  days  after
     transfection  and  was  passed  through  a  0.45-mm  filter
     before  being  stored  at  ¯80°C.   HIV  capsid  protein  (p24)
     levels in the harvested cell culture media were determined
     by an EIA method as described by the manufacturer (SIAC;
35   Frederick,  MD).   Before  infection,  target  cells  (293  and
     293/T)   were   plated   in   cell   culture   media.   Control

infections were performed using cell culture media from mock transfections (no DNA) or transfections containing the fitness test vector plasmid DNA without the envelope expression plasmid. One to three or more days after infection the media was removed and cell lysis buffer (Promega) was added to each well. Cell lysates were assayed for luciferase activity. Alternatively, cells were lysed and luciferase was measured by adding Steady-Glo (Promega) reagent directly to each well without aspirating the culture media from the well.

## Example 11

### Measuring Replication Fitness of Viruses with Deficiencies in Reverse Transcriptase Activity

A means and method is provided for identifying mutations in reverse transcriptase that alter replication fitness. A means and method is provided for identifying mutations that alter replication fitness and can be used to identify mutations associated with other aspects of HIV-1 replication, including, but not limited to integration, virus assembly, and virus attachment and entry. This example also provides a means and method for quantifying the affect that specific mutations reverse transcriptase have on replication fitness. A means and method for quantifying the affect that specfic protease and reverse transcriptase mutations have on replication fitness to mutations in other viral genes involved in HIV-1 replication, including, but not limited to the gag, pol, and envelope genes is also provided.

Fitness test vectors were constructed as described in

example 10. Fitness test vectors derived from patient
samples or clones derived from the fitness test vector
pools, or fitness test vectors were engineered by site
directed mutagenesis to contain specific mutations, and
were tested in a fitness assay to determine accurately and
quantitatively the relative fitness compared to a well-
characterized reference standard.  A patient sample was
examined for increased or decreased reverse transcriptase
activity and correlated with the relative fitness observed
(Figure C).

**Reverse transcriptase activity of patient HIV samples**

Reverse transcriptase activity can be measured by any
number of widely used assay procedures, including but not
limited to homopolymeric extension using (e.g. oligo
dT:poly rC) or real time PCR based on molecular beacons
(reference Kramer) or 5'exonuclease activity (Lie and
Petropoulos, 1996).  In one embodiment, virion associated
reverse transcriptase activity was measured using a
quantitative PCR assay that detects the 5' exonuclease
activity associated with thermo-stable DNA polymerases
(Figure C).  In one embodiment of the invention, the
fitness of the patient virus was compared to a reference
virus to determine the relative fitness compared to
"wildtype" viruses that have not been exposed to reverse
transcriptase inhibitor drugs.  In another embodiment, the
fitness of the patient virus was compared to viruses
collected from the same patient at different timepoints,
for example prior to initiating therapy, before or after
changes in drug treatment, or before or after changes in
virologic (RNA copy number), immunologic (CD4 T-cells), or
clinical (opportunistic infection) markers of disease
progression.

5

**Genotypic analysis of patient HIV samples**

Fitness test vector DNAs, either pools or clones, are analyzed by any of the genotyping methods described in Example 1. In one embodiment of the invention, patient

10 HIV sample sequences were determined using viral RNA purification, RT/PCR and ABI chain terminator automated sequencing. The sequence was determined and compared to reference sequences present in the database or compared to a sample from the patient prior to initiation of therapy.

15 The genotype was examined for sequences that are different from the reference or pre-treatment sequence and correlated to the observed fitness.

**Fitness analysis of site directed mutants**

20

Genotypic changes that are observed to correlate with changes in fitness were evaluated by construction of fitness vectors containing the specific mutation on a defined, wild-type (drug susceptible) genetic background.

25 Mutations may be incorporated alone and/or in combination with other mutations that are thought to modulate the fitness of a virus. Mutations were introduced into the fitness test vector through any of the widely known methods for site-directed mutagenesis. In one embodiment

30 of this invention the mega-primer PCR method for site-directed mutagenesis is used. A fitness test vector containing the specific mutation or group of mutations were then tested using the fitness assay described in Example 10 and the fitness was compared to that of a

35 genetically defined wild-type (drug susceptible) fitness test vector which lacks the specific mutations. Observed

5      changes in fitness are attributed to the specific
       mutations introduced into the resistance test vector. In
       several related embodiments of the invention, fitness test
       vectors containing site directed mutations in reverse
       transcriptase that result in amino acid substitutions at
10     position 190 (G190A, G190S, G190C, G190E, G190V, G190T)
       and that display different amounts of reverse
       transcriptase activity were constructed and tested for
       fitness (Figure D). The fitness results were correlated
       with specific reverse transcriptase amino acid
15     substituions and fitness.


                              **Example 12**
               **Measuring Replication Fitness of Viruses with**
                    **Deficiencies in Protease Activity**

20
       A means and method for identifying mutations in protease
       that alter replication fitness is provided.


       This example provides the means and methods for
25     identifying mutations that alter replication fitness for
       various components of HIV-1 replication, including, but
       not limited to integration, virus assembly, and virus
       attachment and entry. This example also provides a means
       and method for quantifying the affect that specific
30     mutations in protease or reverse transcriptase have on
       replication fitness. This method can be used for
       quantifying the effect that specific protease mutations
       have on replication fitness and can be used to quantify
       the effect of other mutations in other viral genes
35     involved in HIV-1 replication, including, but not limited
       to the gag, pol, and envelope genes.

5

Fitness test vectors were constructed as described in example 10. Fitness test vectors derived from patient samples or clones derived from the fitness test vector pools, or fitness test vectors engineered by site directed
10    mutagenesis to contain specific mutations, were tested in a fitness assay to determine accurately and quantitatively the relative fitness compared to a well-characterized reference standard. A patient sample was examined further for increased or decreased protease activity correlated
15    with the relative fitness observed (Figure C).


**Protease activity of patient HIV samples**

Protease activity can be measured by any number of widely used assay procedures, including but not limited to in
20    vitro reactions that measure protease cleavage activity (reference Erickson ). In one embodiment, protease cleavage of the gag polyprotein (p55) was measured by Western blot analyis using an anti-capsid (p24) antibody (Figure C). In one embodiment of the invention, the
25    fitness of the patient virus was compared to a reference virus to determine the relative fitness compared to "wildtype" viruses that have not been exposed to protease inhibitor drugs. In another embodiment, the fitness of the patient virus was compared to viruses collected from
30    the same patient at different timepoints, for example prior to initiating therapy, before or after changes in drug treatment, or before or after changes in virologic (RNA copy number), immunologic (CD4 T-cells), or clinical (opportunistic infection) markers of disease progression.

35

**Genotypic analysis of patient HIV samples**

5      Fitness test vector DNAs, either pools or clones, are
analyzed by any of the genotyping methods described in
Example 1.   In one embodiment of the invention, patient
HIV sample sequences were determined using viral RNA
purification, RT/PCR and ABI chain terminator automated
10     sequencing.   The sequence was determined and compared to
reference sequences present in the database or compared to
a sample from the patient prior to initiation of therapy,
if available.   The genotype was examined for sequences
that are different from the reference or pre-treatment
15     sequence and correlated to the observed fitness.


**Fitness analysis of site directed mutants**


Genotypic changes that are observed to correlate with
20     changes in fitness are evaluated by construction of
fitness vectors containing the specific mutation on a
defined, wild-type (drug susceptible) genetic background.
Mutations may be incorporated alone and/or in combination
with other mutations that are thought to modulate the
25     fitness of a virus.   Mutations are introduced into the
fitness test vector through any of the widely known
methods for site-directed mutagenesis.   In one embodiment
of this invention the mega-primer PCR method for
site-directed mutagenesis is used.   A fitness test vector
30     containing the specific mutation or group of mutations are
then tested using the fitness assay described in Example
10 and the fitness is compared to that of a genetically
defined wild-type (drug susceptible) fitness test vector
which lacks the specific mutations.   Observed changes in
35     fitness are attributed to the specific mutations
introduced into the fitness test vector.   In several

5      related embodiments of the invention, fitness test vectors
       containing site directed mutations in reverse protease
       that result in amino acid substitutions at positions 30,
       63, 77, 90 (list from Figure E) and that display different
       amounts of protease activity are constructed and tested
10     for fitness (Figure E). The fitness results enable the
       correlation between specific protease amino acid
       substitutions and changes in viral fitness.


                              **Example 13**
15     **Measuring Replication Fitness and Drug Susceptibility in a**
                       **Large Patient Population**


       This example describes the high incidence of patient
       samples with reduced replication fitness. This example
20     also describes the general correlation between reduced
       drug susceptibility and reduced replication fitness. This
       example further describes the occurrence of viruses with
       reduced fitness in patients receiving protease inhibitor
       and/or reverse transcriptase inhibitor treatment. This
25     example further describes the incidence of patient samples
       with reduced replication fitness in which the reduction in
       fitness is due to altered protease processing of the gag
       polyprotein (p55). This example further describes the
       incidence of protease mutations in patient samples that
30     exhibit low, moderate or normal (wildtype) replication
       fitness. This example further describes protease mutations
       that are frequently observed, either alone or in
       combination, in viruses that exhibit reduced replication
       capacity. This example also describes the incidence of
35     patient samples with reduced replication fitness in which
       the reduction in fitness is due to altered reverse

transcriptase activity.    This example describes the occurrence of viruses with reduced replication fitness in patients failing antiretroviral drug treatment.

Fitness/resistance test vectors were constructed as described in example 10.  Fitness and drug susceptibility was measured in 134 random patient samples that were received for routing phenotypic testing by the ViroLogic Clinical Reference Laboratory.  Fitness assays were performed as described in Example 10.  Drug susceptibility testing and genotyping of the protease region was performed as described in Example 1.  Reverse transcriptase activity was measured as described in Example 11.  Protease processing was measured as described in Example 12.

**Drug susceptibility of patient viruses**
Reduced drug susceptibility was observed for a majority of the patient virus samples (Table A).  66 percent of the viruses exhibited large (define as >10X of the reference) reductions in susceptibility to one or more NRTI drugs. 52 percent of the viruses exhibited large reductions in susceptibility to one or more NNRTI drugs. 45 percent of the viruses exhibited large reductions in susceptibility to one or more PRI drugs.

**Fitness of patient viruses**
Reduced replication fitness was observed for a majority of the patient virus samples (Table A). Forty one percent of the viruses exhibited large reductions in replication fitness (<25% of the reference). Another 45% had moderate reductions (between 25-75% of the reference) in

5     replication fitness. A minority of the patient samples (14%) displayed replication fitness that approached or exceeded "wildtype" levels (>75% of the reference). Viruses with reduced drug susceptibility, were much more likely to display reduced replication fitness (Figures F,

10    G, H, and I).

## Protease Mutations in patient viruses

Greater than 10 mutations in protease were observed in a majority of the patient virus samples (Table A). Viruses

15    with reduced fitness were much more likely to contain 10 or more protease mutations (Figure I). Sixty two percent of the viruses that exhibited large reductions in replication fitness (<25% of the reference) contained 10 or more protease mutations. Twenty two percent of the

20    viruses with moderate reductions (between 25-75% of the reference) in fitness contained 10 or more protease mutations. Only 5% of the viruses that displayed replication fitness that approached or exceeded "wildtype" levels (>75% of the reference) contained 10 or more

25    protease mutations (Table A). Certain protease mutations either alone (D30N) or in combination (L90M plus K20T, or M46I, or 73, or N88D) were observed at high incidences in viruses with reduced fitness (Figures I and J).

## Protease processing of patient viruses

30    Reduced protease processing of the p55 gag polyprotein was observed in a majority of the patient virus samples (Table A). Viruses with reduced fitness were much more likely to display reduced protease processing; defined as having

detectable amounts of the p41 intermediate cleavage product (Figures F, I and K). Seventy one percent of the viruses that exhibited large reductions in replication fitness (<25% of the reference) displayed reduced protease processing. Eighteen percent of the viruses with moderate fitness reductions (between 25-75% of the reference) displayed reduced protease processing. Only 10% of the viruses that displayed replication fitness that approached or exceeded "wildtype" levels (>75% of the reference) exhibited reduced protease processing (Table A). Certain protease mutations (D30N, M46I/L, G48V, I54L/A/S/T/V, and I84V) were observed at high incidences in viruses with reduced protease processing of the p55 gag polyprotein (Figure L).

**Reverse transcriptase of patient viruses**

Reduced reverse transcriptase activity processing was observed in a minority of the patient virus samples (Table A). Viruses with reduced fitness were much more likely to display reduced reverse transcriptase activity. Fourteen percent of the viruses that exhibited large reductions in replication fitness (<25% of the reference) displayed reduced reverse transcriptase activity. Only 2% of the viruses with moderate fitness reductions (between 25-75% of the reference) displayed reduced reverse transcriptase activity. None of the viruses that displayed replication fitness that approached or exceeded "wildtype" levels (>75% of the reference) exhibited reduced reverse transcriptase activity.

**Example 14**

**Measuring Replication Fitness to Guide Treatment Decisions**

A means and method for using replication fitness measurements to guide the treatment of HIV-1 is provided.
This example further provides a means and method for using replication fitness measurements to guide the treatment of patients failing antiretroviral drug treatment. This example further provides the means and methods for using replication fitness measurements to guide the treatment of patients newly infected with HIV-1.

**Guiding treatment of patients with multi-drug resistant virus:** Fitness/resistance test vectors were constructed as described in example 10. Fitness and drug susceptibility were measured on serial longitudinal samples collected weekly for 12 weeks from 18 patients. These patients were considered failing a protease inhibitor (typically indinavir) containing regimen and had incomplete suppression of virus replication based on routine viral load testing (>2,500 copies/mL). Phenotypic drug susceptibility testing indicated that these patient viruses were multi-drug resistant. Each patient agreed to interrupt therapy for a period of at least 12 weeks. Phenotypic drug susceptibility assays were performed as described in Example 1 on serial samples collected just prior to interrupting therapy and weekly during the period of interruption. Fitness assays were performed as described in Example 10 on serial samples collected just prior to interrupting therapy and weekly during the period

5    of interruption. Protease processing was measured as
     described in Example 12.

     Of the 18 patients that interrupted therapy, 16 patients
     had resistant viruses that regained susceptibility to
10   antiretroviral drugs during the period of treatment
     interruption. The phenotypic test results of a
     representative patient are shown in Figure M. Typically,
     susceptibility returned to all drug classes
     simultaneously, consistent with the re-emergence of a
15   minor population of drug sensitive virus. In the
     representative example shown in Figure M, drug sensitivity
     was abruptly restored between weeks 9 and 10. Genotypic
     analysis (DNA sequence of protease and reverse
     transcriptase) are also consistent with the re-emergence
20   of a drug sensitive virus. These data show the loss of
     most or all drug resistance mutation simultaneously (data
     not shown). The data are not consistent with random back
     mutations. Back mutations would predict that restored
     susceptibility to drugs would occur unevenly for different
25   drug classes and/or within a drugs within the same class.

     Generally, the re-emergence of the drug susceptible virus
     was also accompanied by a simultaneous increase in
     replication fitness. This relationship is clearly evident
30   for the representative virus (Figure N). Several other
     examples with less frequent timepoints are shown in Figure
     O. Virus from patients that did not revert to drug
     susceptibility after interruption generally did not
     exhibit an increase in replication fitness, nor did

5   viruses from patients that did not interrupt treatment
    (Figures O).  The data indicate that the drug sensitive
    virus that re-emerged after treatment interruption is able
    to replicate better than the drug resistant virus that was
    present before treatment was interrupted.   The re-
10  emergence of drug susceptible virus in this group of
    patients was also accompanied by an increase in viral load
    and a decrease in DC4 T-cells, indicators of disease
    progression.   Thus, fitness information can be used to
    guide treatment of patients that harbor multi-drug
15  resistant virus and are considering treatment
    interruption.  If the patient virus is drug resistant but
    has low replication capacity, the patient and the
    physician should consider continuing drug treatment to
    prevent the re-emergence of a drug sensitive virus with
20  higher replication capacity and greater pathogenecity.
    Alternatively, if the patient virus is drug resistant and
    has high replication capacity, the patient and the
    physician may consider interrupting treatment to spare the
    patient from the harmful and unpleasant side effects of
25  antiretroviral drugs that are not providing clinical
    benefit.

    Furthermore, physicians may choose to perform routine
    replication fitness assays for patients that have multi-
30  drug resistant virus.  This assay could be used to monitor
    the replication fitness of patient viruses when complete
    suppression of virus replication is not possible due to
    multi-drug resistance.   The assay would be used to guide
    treatment decisions that prevent the drug resistant virus

5    with low replication fitness from increasing its
replication fitness. In this way, physicians may prolong
the usefulness of antiretroviral drugs despite the
presence of drug resistant virus in the patient.

10   **Guiding treatment of newly infected patients:**

Patients that maintain high virus loads (setpoint) after
acute infection are more likely to exhibit accelerated
disease progression. Therefore, it is advantageous for
15   this class of patient to initiate antiretroviral drug
treatment as soon as possible after diagnosis with HIV-1
infection. In conjunction with viral load, fitness
measurements of viruses in newly infected patients may
provide a useful measurement to identify those individuals
20   that will develop elevated setpoints after primary
infection and consequently are likely to exhibit
accelerated disease progression. Fitness measurements may
guide the decision to treat immediately after diagnosis or
a some later time point.

25

**Example 15**

**Measuring Saquinavir Susceptibility of Viruses
Containing Various Amino Acid Substitutions in
Protease at Position 82**

30

This example provides a means and method for identifying
mutations in protease that affect susceptibility
(increased or decreased) to saquinavir.

5      In one embodiment of this invention, the effects of
combination of mutations at position 82 (for example,
V82A, V82F, V82S, or V82T are evaluated using the
following method comprising: (i)collecting a biological
sample from an HIV-1 infected subject; (ii) evaluating
10     whether the HIV-1 in the sample contains nucleic acid
encoding protease having a valine to alanine (V82A),
phenylalanine (V82F), serine (V82S), or threonine (V82T)
substitution at position 82 or a leucine to methionine
substitution at position 90 (L90M); and (iii) determining
15     susceptibility to protease inhibitors (PRIs).

The biological sample comprises whole blood, blood
components including peripheral mononuclear cells (PBMC),
serum, plasma (prepared using various anticoagulants such
20     as EDTA, acid citrate-dextrose, heparin), tissue biopsies,
cerebral spinal fluid (CSF), or other cell, tissue or body
fluids. In another embodiment, the HIV-1 nucleic acid
(genomic RNA) or reverse transcriptase protein can be
isolated directly from the biological sample or after
25     purification of virus particles from the biological
sample. Evaluating whether the amino acid at position 82
of the HIV-1 protease is mutated to alanine,
phenylalanine, or threonine, can be performed using
various methods, such as direct characterization of the
30     viral nucleic acid encoding protease or direct
characterization of the protease protein itself. Defining
the amino acid at position 82 of protease can be performed
by direct characterization of the protease protein by
conventional or novel amino acid sequencing methodologies,

5    epitope recognition by antibodies or other specific
binding proteins or compounds. Alternatively, the amino
acid at position 82 of the HIV-1 protease protein can be
defined by characterizing amplified copies of HIV-1
nucleic acid encoding the protease protein. Amplification

10   of the HIV-1 nucleic acid can be performed using a variety
of methodologies including reverse
transcription-polymerase chain reaction (RT-PCR), NASBA,
SDA, RCR, or 3SR. The nucleic acid sequence encoding HIV
protease at codon 82 can be determined by direct nucleic

15   acid sequencing using various primer extension-chain
termination (Sanger, ABI/PE and Visible Genetics) or chain
cleavage (Maxam and Gilbert) methodologies or more
recently developed sequencing methods such as matrix
assisted laser desorption-ionization time of flight

20   (MALDI-TOF) or mass spectrometry (Sequenom, Gene Trace
Systems). Alternatively, the nucleic acid sequence
encoding amino acid position 82 can be evaluated using a
variety of probe hybridization methodologies, such as
genechip hybridization sequencing (Affymetrix), line probe

25   assay (LiPA; Murex), and differential hybridization
(Chiron).


In a preferred embodiment of this invention, evaluation of

30   the effects of mutations at amino acid position 82 of
HIV-1 protease on protease inhibitor susceptibility, was
carried out using a phenotypic susceptibility assay using
resistance test vector DNA prepared from the biological
sample. In one embodiment, plasma samples were collected,

5      viral RNA was purified and an RT-PCR methodology was used
       to amplify a patient derived segment encoding the HIV-1
       protease and reverse transcriptase regions. The amplified
       patient derived segments were then incorporated, via DNA
       ligation and bacterial transformation, into an indicator
10     gene viral vector thereby generating a resistance test
       vector. Resistance test vector DNA was isolated from the
       bacterial culture and the phenotypic susceptibility assay
       was carried out as described in Example 1. The genotype of
       the protease region was determined by dideoxy chain-
15     termination sequencing of the resistance test vector DNA.
       The results are summarized for saquinavir (SQV) in Figure
       6. Samples were categorized as having mutations in
       protease encoding alanine (A), phenylalanine (F), or
       threonine (T) at position 82, instead of the wild-type
20     valine (V), and the percentage of samples in each category
       displaying hyper-sensitivity to saquinavir (i.e., fold-
       change vs. reference of 0.4 or less) was determined.
       Surprisingly, the percentage of saquinavir hyper-
       susceptible viruses was much higher amongst viruses
25     containing V82F than those containing V82A or V82T. This
       observation implies that the detection of V82F in protease
       predicts a positive virological response to saquinavir
       treatment.

5

## Example 16
### Measuring Replication Fitness of Viruses with
### Mutations in Integrase

10    This example provides a means and method for identifying
mutations in integrase that alter replication fitness.

This example provides the means and methods for
identifying mutations that alter replication fitness for
15    various components of HIV-1 replication, including, but
not limited to integration, virus assembly, and virus
attachment and entry.  This example also provides a means
and method for quantifying the affect that specific
mutations in protease, reverse transcriptase, or integrase
20    have on replication fitness.  This method can be used for
quantifying the effect that specific integrase mutations
have on replication fitness and can be used to quantify
the effect of other mutations in other viral genes
involved in HIV-1 replication, including, but not limited
25    to the gag, pol, and envelope  genes.

Fitness test vectors engineered by site directed
mutagenesis to contain specific mutations in integrase
were tested in a fitness assay to determine accurately and
30    quantitatively the relative fitness compared to a well-
characterized reference standard.

Genotypic changes that are observed to correlate with
resistance to integrase inhibitors are evaluated by

5　　construction of fitness vectors containing the specific
mutation on a defined, wild-type (drug susceptible)
genetic background.　Mutations may be incorporated alone
and/or in combination with other mutations that are
thought to modulate the　fitness of a virus.　Mutations

10　　are introduced into the fitness test vector through any of
the widely known methods for site-directed mutagenesis. In
one embodiment of this invention the mega-primer PCR
method for site-directed mutagenesis is used (Sarkar, G.
and Sommar, S.S., 1994, Biotechniques 8, 404-407).　A

15　　fitness test vector containing the specific mutation or
group of mutations are then tested using the fitness assay
described in Example 10 and the fitness is compared to
that of a genetically defined wild-type (drug susceptible)
fitness test vector which lacks the specific mutations.

20　　Observed changes in fitness are attributed to the specific
mutations introduced into the fitness test vector.　In
several related embodiments of the invention, fitness test
vectors containing site directed mutations in integrase
that result in amino acid substitutions at positions 66,

25　　154, 66 and 153, and 66 and 154 are constructed and tested
for fitness (Figure 7).　As controls, mutants with
multiple changes conferring resistance to reverse
transcriptase and protease inhibitors (MDRC4) and with a
mutation in the integrase active site (D64V) were also

30　　tested.　The fitness results enable the correlation
between specific integrase amino acid substitutions and
changes in viral fitness.

5    Figure 6. Distribution of saquinavir hyper-susceptibility
     by amino acid change at position 82.



10

5

Figure 7. Relative luciferase activity of integrase
inhibitor-resistant site-directed mutants.



| | NL4-3 | MDRC4 | D64V | T66I | M154I | 66/153 | 66/154 |
|---|---|---|---|---|---|---|---|
| ∎ avg. RLU | 100 | 21 | 0.03 | 5 | 42 | 31 | 86 |
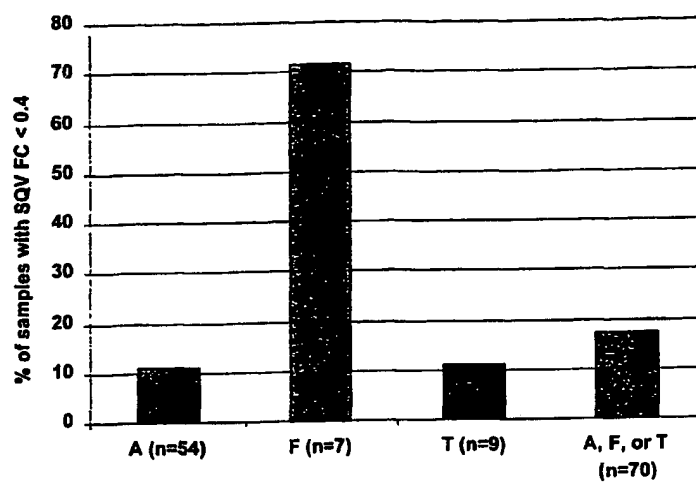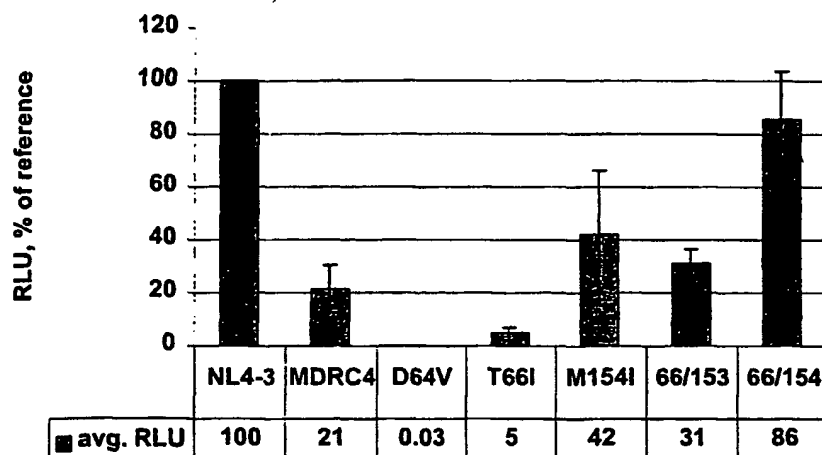
10

15

●

●

5

10

15

20

25 Table 1:  PRI susceptibility of selected patient samples.
Viruses displaying increased susceptibility to amprenavir
(5-fold or greater) were genotyped and found to contain
the N88S mutation in PR.  Samples were listed in order of
decreasing amprenavir susceptibility.

**Table 1**

| Sample ID | Prior PRI Experience | Fold Change vs. Reference | | | | | PR Mutations |
|---|---|---|---|---|---|---|---|
| | | SQV | IDV | RTV | NFV | AMP | |
| 0732 | NFV | 0.73 | 2.11 | 1.72 | 8.93 | 0.08 | K14R, II5V, K20T, E35D, M36I, R41K, I62V, L63Q, N88S |
| 627 | IDV | 0.26 | 6.16 | 1.50 | 21.06 | 0.09 | II3I/V, E35D, M46L, L63P, I64V, I73V, N88S |
| 1208 | NFV | 1.55 | 3.15 | 1.22 | 11.16 | 0.10 | I62V, L63P, V77I, N88S |
| 360 | IDV | 1.88 | 6.31 | 1.49 | 29.95 | 0.15 | II3V, K20M, M36V, N37A, M46I, I62V, L63P, N88S, I93L |
| 0910 | NFV | 1.41 | 5.47 | 1.85 | 16.76 | 0.16 | M46I, L63P, V77I, N88S, I93I/L |
| 3542 | IDV | 1.28 | 7.61 | 3.16 | 24.67 | 0.16 | II3V, K14R, N37D, M46I, L63P, N88S, I93L |
| 3654 | | 1.80 | 7.56 | 1.95 | 18.61 | 0.20 | II3V, R41K, M46I, L63P, V77I, N88S, I93L |

Fold Change Limits: >2.5  <0.4

5　　　Table 2:　PRI susceptibility of site-directed mutants in
　　　PR.　Mutations were introduced into the drug sensitive
　　　reference resistance test vector and the susceptibility to
　　　PRIs was determined.

**Table 2**

| Site-Directed Mutations | Fold Change vs. reference | | | | |
| --- | --- | --- | --- | --- | --- |
| | SQV | IDV | RTV | NFV | AMP |
| L63P | 1.04 | 1.12 | 1.27 | 1.43 | 1.06 |
| L63P, V77I | 1.24 | 1.72 | 1.73 | 2.49 | 0.91 |
| N88S | 0.47 | 1.56 | 0.36 | 2.39 | 0.04 |
| L63P, N88S | 1.44 | 2.56 | 0.77 | 5.10 | 0.11 |
| L63P, V77I, N88S | 1.24 | 3.09 | 1.39 | 12.59 | 0.08 |
| M46L, L63P, N88S | 1.15 | 2.30 | 0.85 | 8.18 | 0.12 |
| M46L, L63P, V77I, N88S | 1.45 | 2.97 | 1.33 | 12.21 | 0.14 |

FOLD CHANGE LIMITS:　<0.4　>2.5

5

Table 3: Relative luciferase activity levels for patient
sample virus-derived resistance test vector pools. The
luciferase activity (relative light units, RLU) measured
in the absence of drug for the patient sample was compared
10  to that of the drug sensitive reference control from the
same assay run, and expressed as a percentage of control.
These values are from one assay each. All the samples
that contain the N88S mutations in PR were found to have
reduced luciferase activity compared to control.

15

**Table 3**

| Sample ID | PR Mutations | Relative Luciferase Activity (% of control) |
|---|---|---|
| 0732 | K14R, I15V, K20T, E35D, M36I, R41K, I62V, L63Q, N88S | 8.5 |
| 627 | I13I/V, E35D, M46L, L63P, I64V, I73V, N88S | 0.7 |
| 1208 | I62V, L63P, V77I, N88S | 14.2 |
| 360 | I13V, K20M, M36V, N37A, M46I, I62V, L63P, N88S, I93L | 2.2 |
| 0910 | M46I, L63P, V77I, N88S, I93I/L | 16.0 |
| 3542 | I13V, K14R, N37D, M46I, L63P, N88S, I93L | 4.6 |
| 3654 | I13V, R41K, M46I, L63P, V77I, N88S, I93L | 12.8 |

5

Table 4: Relative luciferase activity levels for
resistance test vectors containing site-directed
mutations. The luciferase activity (relative light units,
RLU) measured in the absence of drug for the mutant was
10 compared to that of the drug sensitive reference control
from the same assay run, and expressed as a percentage of
control. These values are from one to five assays each,
and each value was obtained using an independent clone for
mutants which were tested multiple times. All the
15 constructs that contain the N88S mutations in PR were
found to have reduced luciferase activity compared to
control. All the constructs with the K20T mutation were
essentially inactive in the assay.

**Table 4**

| Site-Directed Mutations | Average Luciferase Activity (% of control) | number of clones tested |
|---|---|---|
| L63P | 163.9 | 1 |
| L63P, V77I | 75.6 | 1 |
| N88S | 1.0 | 3 |
| L63P, N88S | 20.7 | 2 |
| L63P, V77I, N88S | 29.3 | 2 |
| M46L, L63P, N88S | 28.0 | 2 |
| M46L, L63P, V77I, N88S | 53.2 | 5 |
| K20T, N88S | <0.01 | 5 |
| K20T, L63P, N88S | <0.01 | 1 |

Table 5:  Oligonucleotide primers used for PCR
amplification and for generating site-directed mutants.

**Table 5.**

| Primer name: | | |
|---|---|---|
| #1): PCR6 | 5' | CCAATTRYTGTGATATTTCTCATGNTCHTCTTGGG          3' (35-mer) |
| #2: PDS/Aps | 5' | CATGTTGCAGGGCCCCTAGGĂAAAAGGGCTGTTGGAAATGTG 3' (42-mer) |
| #3: PDS/Age | 5' | CACTCCATGTACCGGTTCTTTTAGAATYTCYCTG          3' (34-mer) |
| #4: RsrII | 5' | ACTTTCGGACCGTCCATTCCTGGCTTTAATTTTACTGGTACAG 3' (43-mer) |
| #5: K20T | 5' | GGGGGGCAATTAACGGAAGCTCTATTAG          3' (28-mer) |
| #6: M46L | 5' | GATGGAAACCAAAATTGATAGGGGGAATTG          3' (30-mer) |
| #7: L63P | 5' | GTATGATCAGATACCCATAGAAATCTGC          3' (28-mer) |
| #8: N88S | 5' | CTGAGTCAACAGACTTCTTCCAATTATG          3' (28-mer) |

R = A or G
Y = C or T
N = A, C, G, or T
H = A, C, or T

**Table 6. PRI Susceptibility (Fold Change <2.5) of Viruses
with Mutations at 82 and/or 90**

| | Percent of viruses with indicated primary mutation(s) which are drug sensitive (fold change in IC50 < 2.5) | | |
|---|---|---|---|
| drug | V82A/F/S/T | L90M | V82A/F/S/T and L90M |
| RTV | 8.0 | 27.6 | 3.0 |
| NFV | 20.0 | 8.6 | 3.0 |
| IDV | 22.7 | 31.0 | 9.1 |
| AMP | 53.3 | 65.5 | 33.3 |
| SQV | 73.3 | 46.6 | 21.2 |

5

Table 7. Correlation Between 82A/F/S/T, Secondary
Mutations, and IDV Susceptibility.

| position | n | % FC > 2.5 | chi square p |
|---|---|---|---|
| 24 | 20 | 100% | <0.005 |
| 71 | 27 | 100% | <0.0001 |
| 54 | 38 | 95% | <0.0001 |
| 46 | 35 | 89% | <0.01 |
| 10 | 47 | 83% | <0.05 |
| 63 | 72 | 79% | <0.05 |
| 82 | 75 | 77% | |

all virus with V82A/F/S/T and no other primary mutations.

Table 8. Correlation Between 82A/F/S/T, Secondary
Mutations, and SQV Susceptibility.

| position | n | % FC > 2.5 | chi square p |
|---|---|---|---|
| 20 | 5 | 80% | <0.001 |
| 36 | 11 | 73% | <0.001 |
| 24 | 20 | 65% | <0.0001 |
| 71 | 27 | 52% | <0.0001 |
| 54 | 38 | 47% | <0.0001 |
| 10 | 47 | 40% | <0.001 |
| 82 | 75 | 27% | · |

**all virus**

Table 9. Association Between SQV and IDV Susceptibility,
V82A/F/S/T, and Number of Resistance Associated Mutations

| Number of secondary mutations | Number of samples | % with IDV FC > 2.5 | % with SQV FC > 2.5 |
|---|---|---|---|
| 1 | 75 | 77 | 27 |
| 2 | 67 | 82 | 30 |
| 3 | 51 | 88 | 39 |
| 4 | 38 | 95 | 50 |
| 5 | 25 | 96 | 60 |
| 6 | 17 | 100 | 76 |
| 7 | 5 | 100 | 60 |

5

Table 10. Correlation Between L90M, Secondary
Mutations, and IDV Susceptibility.

| position | n | % FC > 2.5 | chi square p |
|---|---|---|---|
| 73 | 19 | 89% | <0.01 |
| 71 | 18 | 89% | <0.001 |
| 46 | 25 | 88% | <0.05 |
| 90 | 58 | 69% | |

15

all viruses with L90M and

Table 11. Correlation Between L90M, Secondary
Mutations, and SQV Susceptibility.

| position | n | % FC > 2.5 | chi square p |
|---|---|---|---|
| 73 | 19 | 79% | <0.01 |
| 71 | 18 | 78% | <0.001 |
| 77 | 25 | 76% | <0.05 |
| 10 | 34 | 65% | <0.05 |
| 90 | 58 | 55% | |

all viruses

Table 12. Association Between SQV and IDV Susceptibility, L90M, and Number of Resistance Associated Mutations.

| Number of secondary mutations | Number of samples | % w with IDV FC > 2.5 | % w with SQV FC > 2.5 |
|---|---|---|---|
| 0 | 58 | 69 | 53 |
| 1 | 57 | 70 | 47 |
| 2 | 56 | 70 | 48 |
| 3 | 41 | 80 | 68 |
| 4 | 31 | 87 | 77 |
| 5 | 14 | 100 | 100 |
| 6 | 6 | 100 | 100 |

-163-

## Summary of the Invention

In another embodiment of this invention, a method is provided of assessing the effectiveness of protease antiretroviral therapy of an HIV-infected patient comprising:

5

(a) collecting a biological sample from the HIV-infected patient;

(b) evaluating whether the biological sample contains nucleic acid encoding HIV protease having a mutation at codon 82 and a secondary mutation at codons selected from the group consisting of 84, 48, 23, 73, 53, 33, 74, 20, 90, 32 and 39 or a mutation at codon 90 and a secondary mutation at codons selected from the group consisting of 53, 66, 84, 54, 48, 33, 73, 20, 71, 64 and 93, and

10

15

(c) determining a change in susceptibility to a protease inhibitor, wherein the protease inhibitor is saquinavir.

20

In another embodiment of this invention, the above method is provided of assessing the effectiveness of protease antiretroviral therapy, having a mutation at codon 82 and a secondary mutation at codons selected from the group consisting of 84, 48, 23, 73, 53, 33, 74, 20, and 90, or a mutation at codon 90 and a secondary mutation at codons selected from the group consisting of 53, 66, 84, 54, 48, 33, 73, 20, and 71, wherein the change in susceptibility in step (c) is a decrease in susceptibility to saquinavir.

25

30

In another embodiment of this invention, the above method is provided of assessing the effectiveness of protease antiretroviral therapy, having a mutation at codon 82 and

a secondary mutation at codons 32 or 39, or a mutation at codon 90 and a secondary mutation at codons 64 or 93, wherein the change in susceptibility in step (c) is an increase in susceptibility to saquinavir.

In another embodiment of this invention, the above method is provided of assessing the effectiveness of protease antiretroviral therapy, having a mutation at codon 90 and a secondary mutation at codons selected from the group consisting of 53, 95, 54, 84, 82, 46, 13, and 74, wherein the protease inhibitor is indinavir.

In another embodiment of this invention, the above method is provided of assessing the effectiveness of protease antiretroviral therapy, having a mutation at codon 90 and a secondary mutation at codons selected from the group consisting of 53, 95, 54, 84, 82, and 46, wherein the change in susceptibility in step (c) is a decrease in susceptibility to indinavir.

In another embodiment of this invention, the above method is provided of assessing the effectiveness of protease antiretroviral therapy, having a mutation at codon 90 and a secondary mutation at codons 13 or 74, wherein the change in susceptibility in step (c) is an increase in susceptibility to indinavir.

In another embodiment of this invention, the above method is provided of assessing the effectiveness of protease antiretroviral therapy, having a mutation at codon 82 and a secondary mutation at·codons selected from the group consisting of 73, 55, 48, 20, 43, 53, 90, 13, 48, 23, 84, 53, 74, 60, 33, 36, 35, 32, and 46 or a mutation at codon

90 and a secondary mutation at codons selected from the group consisting of 95, 55, 54, 82, 85, 84, 20, 72, 62, 74, 53, 48, 23, 58, 36, 64, 77, and 93.

In another embodiment of this invention, the above method is provided of assessing the effectiveness of protease antiretroviral therapy, wherein the protease inhibitor is selected from the group consisting of indinavir, amprenavir, and saquinavir.

In another embodiment of this invention, the above method is provided of assessing the effectiveness of protease antiretroviral therapy, wherein step (c) is determining a change in susceptibility to the protease inhibitor greater than 10 fold.

In another embodiment of this invention, the above method is provided of assessing the effectiveness of protease antiretroviral therapy, having a mutation at codon 82 and a secondary mutation at codons selected from the group consisting of 48, 23, 84, 53, 74, 20, 60, 33, 36, 35, or a mutation at codon 90 and a secondary mutation at codons selected from the group consisting of 84, 53, 48, 23, 58, 20, 36, and 54, wherein the change in susceptibility in step (c) is a decrease in susceptibility to saquinavir.

In another embodiment of this invention, the above method is provided of assessing the effectiveness of protease antiretroviral therapy, having a mutation at codon 82 and a secondary mutation at codons 32 or 46, or a mutation at codon 90 and a secondary mutation at codons 64, 77, or 93, wherein the change in susceptibility in step (c) is an increase in susceptibility to saquinavir.

In another embodiment of this invention, the above method is provided of assessing the effectiveness of protease antiretroviral therapy, having a mutation at codon 82 and a secondary mutation at codons selected from the group consisting of 73, 55, 48, 20, 43, 53, and 90, or a mutation at codon 90 and a secondary mutation at codons selected from the group consisting of 95, 55, 54, 82, 85, 84, 20, 72, and 62, wherein the change in susceptibility in step (c) is a decrease in susceptibility to indinavir.

In another embodiment of this invention, the above method is provided of assessing the effectiveness of protease antiretroviral therapy, having a mutation at codon 82 and a secondary mutation at codon 13, or a mutation at codon 90 and a secondary mutation at codon 74, wherein the change in susceptibility in step (c) is an increase in susceptibility to indinavir.

In another embodiment of this invention, a method is provided of assessing the effectiveness of protease antiretroviral therapy of an HIV-infected patient comprising:

(a)  collecting a biological sample from the HIV-infected patient;

(b)  evaluating whether the biological sample contains nucleic acid encoding HIV protease having a mutation at codon 90 and secondary mutations of at least three codons; and

(c)  determining a decrease in susceptibility to saqinavir.

In another embodiment of this invention, the above method is provided of assessing the effectiveness of protease

antiretroviral therapy, wherein in the evaluating step (b), the nucleic acid encoding HIV protease has secondary mutations of at least five codons.

5    In another embodiment of this invention, the above method is provided of assessing the effectiveness of protease antiretroviral therapy, wherein the secondary mutation are selected from the group consisting of codons 10, 20, 52, 53, 54, 66, 71, 73 and 84.

10

In another embodiment of this invention, a method is provided of assessing the effectiveness of protease antiretroviral therapy of an HIV-infected patient comprising:

15    (a)    collecting a biological sample from the HIV-infected patient;

      (b)    evaluating whether the biological sample contains nucleic acid encoding HIV protease having a mutation at codon 82 and secondary

20           mutations at codons selected from the group consisting of 33, 23, 84, 32, 53, 90, 37, 71, 46, 10, 54, 61, 11, and 46, or a mutation at codon 90 and secondary mutations at codons selected from the group consisting of 89, 53,

25           84, 33, 92, 95, 54, 58, 46, 82, 36, 10, 62, 74, 15, 47, 66, 32, 55, 53, 13, and 69; and

      (c)    determining a change in susceptibility to amprenavir.

30    In another embodiment of this invention, the above method is provided of assessing the effectiveness of protease antiretroviral therapy, wherein the mutation at codon 82 is a substitution of alanine (A), phenylalanine (F),

serine (S), or threonine (T) for valine(V) and the mutation at codon 90 is a substitution of methionine (M) for leucine (L).

5     In another embodiment of this invention, the above method is provided of assessing the effectiveness of protease antiretroviral therapy, having a mutation at codon 82 and secondary mutations at codons selected from the group consisting of 33, 23, 84, 32, 53, 90, 37, 71, 46, 10, 54,
10     11, and 46, or a mutation at codon 90 and secondary mutations at codons selected from the group consisting of 89, 53, 84, 33, 92, 95, 54, 58, 46, 82, 36, 10, 62, 47, 66, 32, 55, 53, and 13; wherein the change in susceptibility in step (c) is a decrease in susceptibility
15     to saquinavir.

    In another embodiment of this invention, the above method is provided of assessing the effectiveness of protease antiretroviral therapy, having a mutation at codon 82 and
20     a secondary mutation at codon 61, or a mutation at codon 90 and secondary mutations at codons 74, 15, or 69, wherein the change in susceptibility in step (c) is an increase in susceptibility to saquinavir.

25     In another embodiment of this invention, a resistance test vector is provided comprising an HIV patient-derived segment comprising nucleic acid encoding protease having a mutation at codon 82 and secondary mutations at codons selected from the group consisting of 73, 55, 48, 20, 43,
30     53, 90, 13, 84, 23, 33, 74, 32, 39, 60, 36, and 35, or a mutation at codon 90 and secondary mutations at codons selected from the group consisting of 53, 95, 54, 84, 82, 46, 13, 74, 55, 85, 20, 72, 62, 66, 84, 48, 33, 73, 71,

64, 93, 23, 58, and 36 and an indicator gene, wherein the expression of the indicator gene is dependent upon the patient-derived segment.

In another embodiment of this invention, the above resistance test vector is provided, wherein the mutation of the patient derived segment at codon 82 is a substitution of alanine (A), phenylalanine (F), serine (S), or threonine (T) for valine(V) and the mutation at codon 90 is a substitution of methionine (M) for leucine (L).

**Phenotypic Susceptibility:**

Phenotypic assays provide information relating to drug resistance in the form of a fold-change in IC50 value, i.e. the ratio of the IC50 for the patient virus to that of a drug sensitive reference control. The significance of the fold change value with respect to treatment choices is limited by at least two factors: the reproducibility of the assay, and the achievable drug concentration at the site of action in the patient. For the PhenoSense™ assay described herein, the reproducibility cut-off is 2.5-fold. For most protease inhibitors, the level of reduction in susceptibility required to overcome the achievable plasma drug concentration is not well defined. However retrospective clinical studies using the 2.5-fold cutoff have suggested that this value is useful for predicting response to protease inhibitors, at least when used alone or in combination with reverse transcriptase inhibitors. Recently, the use of dual protease inhibitor based regimens (typically involving co-dosing of an inhibitor with ritonavir or nelfinavir) has become popular, since the plasma drug levels can be significantly boosted due to

inhibition of metabolic pathways. In cases such as these,
it is likely that the clinically relevant fold-change
cutoff will be higher, perhaps 10-fold. Future clinical
studies will be required in order to accurately determine

5     the actual clinical cutoff value.


As used herein, what it is understood to mean "secondary
mutations" in addition to the discussion on pages 7 and 8
of this specification, is that other mutations, not

10    currently recognized as resistance-associated, may also be
defined as "secondary mutations" if they enhance the
effects of primary mutations.


## Example 17

15    **Predicting Response to Protease Inhibitors by
Characterization of Amino Acid 82 of HIV-1 Protease.**


In one embodiment of this invention, changes in the amino
acid at position 82 of the protease protein of HIV-1 are

20    evaluated using the following method comprising: (i)
collecting a biological sample from an HIV-1 infected
subject; (ii) evaluating whether the biological sample
contains nucleic acid encoding HIV-1 protease having a
valine to alanine (V82A), phenylalanine (V82F), serine

25    (V82S), threonine (V82T), or other amino acid substitution
at codon 82 ("V82 mutations"); and (iii) determining
susceptibility to protease inhibitors (PRI).


The biological sample comprises whole blood, blood

30    components including peripheral mononuclear cells (PBMC),
serum, plasma (prepared using various anticoagulants such
as EDTA, acid citrate-dextrose, heparin), tissue biopsies,
cerebral spinal fluid (CSF), or other cell, tissue or body

fluids.   In another embodiment, the HIV-1 nucleic acid (genomic RNA) or reverse transcriptase protein can be isolated directly from the biological sample or after purification of virus particles from the biological sample.  Evaluating whether the amino acid at position 82 of the HIV-1 protease is mutated to alanine, phenylalanine, serine, threonine, or other amino acids, can be performed using various methods, such as direct characterization of the viral nucleic acid encoding protease or direct characterization of the protease protein itself.  Defining the amino acid at position 82 of protease can be performed by direct characterization of the protease protein by conventional or novel amino acid sequencing methodologies, epitope recognition by antibodies or other specific binding proteins or compounds.  Alternatively, the amino acid at position 82 of the HIV-1 protease protein can be defined by characterizing amplified copies of HIV-1 nucleic acid encoding the protease protein.  Amplification of the HIV-1 nucleic acid can be performed using a variety of methodologies including reverse transcription-polymerase chain reaction (RT-PCR), NASBA, SDA, RCR, or 3SR. The nucleic acid sequence encoding HIV protease at codon 82 can be determined by direct nucleic acid sequencing using various primer extension-chain termination (Sanger, ABI/PE and Visible Genetics) or chain cleavage (Maxam and Gilbert) methodologies or more recently developed sequencing methods such as matrix assisted laser desorption-ionization time of flight (MALDI-TOF) or mass spectrometry (Sequenom, Gene Trace Systems). Alternatively, the nucleic acid sequence encoding amino acid position 82 can be evaluated using a variety of probe hybridization methodologies, such as genechip

hybridization sequencing (Affymetrix), line probe assay
(LiPA; Murex), and differential hybridization (Chiron).

In a preferred embodiment of this invention, evaluation of
protease inhibitor susceptibility and of whether amino
acid position 82 of HIV-1 protease was wild type or mutant
was carried out using a phenotypic susceptibility assay or
genotypic assay, respectively, using resistance test
vector DNA prepared from the biological sample. In one
embodiment, plasma sample was collected, viral RNA was
purified and an RT-PCR methodology was used to amplify a
patient derived segment encoding the HIV-1 protease and
reverse transcriptase regions. The amplified patient
derived segments were then incorporated, via DNA ligation
and bacterial transformation, into an indicator gene viral
vector thereby generating a resistance test vector.
Resistance test vector DNA was isolated from the bacterial
culture and the phenotypic susceptibility assay was
carried out and analyzed as described in Example 1.

The nucleic acid (DNA) sequence of the patient derived
HIV-1 protease and reverse transcriptase regions was
determined using a fluorescence detection chain
termination cycle sequencing methodology (ABI/PE). The
method was used to determine a consensus nucleic acid
sequence representing the combination of sequences of the
mixture of HIV-1 variants existing in the subject sample
(representing the quasispecies), and to determine the
nucleic acid sequences of individual variants. Genotypes
are analyzed as lists of amino acid differences between
virus in the patient sample and a reference laboratory
strain of HIV-1, NL4-3. Genotypes and corresponding
phenotypes (fold-change in IC50 values) are entered in a

-173-

relational database linking these two results with patient information. Large datasets can then be assembled from patient virus samples sharing particular characteristics, such as the presence of any given mutation or reduced
5  susceptibility to any drug or combination of drugs.

**(a) Protease inhibitor susceptibility of viruses containing mutations at amino acid 82 of HIV-1 Protease.**

10  Phenotypic susceptibility profiles of 270 patient virus samples that contained a mutation at position 82 (but not at positions 30 or 50, which are primary mutations associated with resistance to nelfinavir and amprenavir, respectively) were analyzed. According to most published
15  guidelines, such viruses are expected to be resistant to ritonavir, nelfinavir, indinavir, and saquinavir. However, only 61.7% of these samples displayed reduced susceptibility to saquinavir using a 2.5-fold threshold (Table 13), while 31.2% and 40.0% displayed reduced
20  susceptibility to saquinavir and indinavir, respectively, using a 10-fold threshold (Table 14). Thus, there was poor correlation between the presence of mutations at position 82 and saquinavir or indinavir susceptibility.

25  **(b) Indinavir susceptibility (fold change threshold 10) of viruses containing combinations of mutations at amino acid 82 and one secondary mutation in HIV-1 Protease.**

To explore the possibility that indinavir resistance (fold
30  change in IC50 > 10-fold) in viruses containing mutations at position 82 requires the presence of other specific mutations, decreased indinavir susceptibility (fold-change in IC50 greater than 10) in viruses containing V82

mutations was correlated with the presence of mutations at other positions. This analysis revealed several positions (most strongly 73, 55, 48, 20, 43, 53, and 90) that decreased indinavir susceptibility significantly in combination with V82 mutations, compared to when these other mutations were absent (see Table 15). The presence of a mutation at position 13 significantly decreased the proportion of samples that had reduced indinavir susceptibility (45.9% vs. 62.2%; Table 15). In other words, the absence of a mutation at position 13 was correlated with decreased susceptibility to indinavir.

**(c) Saquinavir susceptibility (fold change threshold 2.5) of viruses containing combinations of mutations at amino acid 82 and one secondary mutation in HIV-1 Protease.**

To explore the possibility that saquinavir resistance in viruses containing mutations at position 82 requires the presence of other specific mutations, decreased saquinavir susceptibility (fold-change in IC50 greater than 2.5-fold) in viruses containing V82 mutations was correlated with the presence of mutations at other positions. This analysis revealed that several positions (most strongly 84, 48, 23, 73, 53, 33, 74, 20, and 90) were associated with reduced saquinavir susceptibility (See Table 16). The combination of mutations at position 82 with a mutation at position 32 or 39 significantly decreased the proportion of samples that had reduced saquinavir susceptibility (Table 16). In other words, the absence of a mutation at position 32 or 39 was correlated with decreased susceptibility to indinavir.

**(d) Saquinavir susceptibility (fold change threshold 10) of viruses containing combinations of mutations at amino acid 82 and one secondary mutation in HIV-1 Protease.**

5      To explore the possibility that saquinavir resistance in viruses containing mutations at position 82 requires the presence of other specific mutations, decreased saquinavir susceptibility (fold-change in IC50 greater than 10 fold) in viruses containing V82 mutations was correlated with
10     the presence of mutations at other positions. This analysis revealed that several positions (most strongly 48, 23, 84, 53, 74, 20, 60, 33, 36, 35, and 90) were associated with reduced saquinavir susceptibility (See Table 17). The combination of mutations at position 82
15     with a mutation at position 32 or 46 significantly decreased the proportion of samples that had reduced saquinavir susceptibility (Table 17). In other words, the absence of a mutation at position 32 or 46 was correlated with decreased susceptibility to indinavir.

20

**Example 18**

**Predicting Response to Protease Inhibitors by Characterization of Amino Acid 90 of HIV-1 Protease.**

25     In one embodiment of this invention, changes in the amino acid at position 90 of the protease protein of HIV-1 are evaluated using the following method comprising: (i) collecting a biological sample from an HIV-1 infected subject; (ii) evaluating whether the biological sample
30     contains nucleic acid encoding HIV-1 protease having a leucine to methionine (L90M) substitution at codon 90; and (iii) determining susceptibility to protease inhibitors (PRI).

●                    ●

The biological sample comprises whole blood, blood components including peripheral mononuclear cells (PBMC), serum, plasma (prepared using various anticoagulants such as EDTA, acid citrate-dextrose, heparin), tissue biopsies,

5    cerebral spinal fluid (CSF), or other cell, tissue or body fluids. In another embodiment, the HIV-1 nucleic acid (genomic RNA) or reverse transcriptase protein can be isolated directly from the biological sample or after purification of virus particles from the biological

10   sample. Evaluating whether the amino acid at position 90 of the HIV-1 protease is mutated to methionine, can be performed using various methods, such as direct characterization of the viral nucleic acid encoding protease or direct characterization of the protease

15   protein itself. Defining the amino acid at position 90 of protease can be performed by direct characterization of the protease protein by conventional or novel amino acid sequencing methodologies, epitope recognition by antibodies or other specific binding proteins or

20   compounds. Alternatively, the amino acid at position 90 of the HIV-1 protease protein can be defined by characterizing amplified copies of HIV-1 nucleic acid encoding the protease protein. Amplification of the HIV-1 nucleic acid can be performed using a variety of

25   methodologies including reverse transcription-polymerase chain reaction (RT-PCR), NASBA, SDA, RCR, or 3SR. The nucleic acid sequence encoding HIV protease at codon 90 can be determined by direct nucleic acid sequencing using various primer extension-chain termination (Sanger, ABI/PE

30   and Visible Genetics) or chain cleavage (Maxam and Gilbert) methodologies or more recently developed sequencing methods such as matrix assisted laser desorption-ionization time of flight (MALDI-TOF) or mass

-177-

spectrometry (Sequenom, Gene Trace Systems). Alternatively, the nucleic acid sequence encoding amino acid position 90 can be evaluated using a variety of probe hybridization methodologies, such as genechip hybridization sequencing (Affymetrix), line probe assay (LiPA; Murex), and differential hybridization (Chiron).

In a preferred embodiment of this invention, evaluation of protease inhibitor susceptibility and of whether amino acid position 90 of HIV-1 protease was wild type or methionine, was carried out using a phenotypic susceptibility assay or genotypic assay, respectively, using resistance test vector DNA prepared from the biological sample. In one embodiment, plasma sample was collected, viral RNA was purified and an RT-PCR methodology was used to amplify a patient derived segment encoding the HIV-1 protease and reverse transcriptase regions. The amplified patient derived segments were then incorporated, via DNA ligation and bacterial transformation, into an indicator gene viral vector thereby generating a resistance test vector. Resistance test vector DNA was isolated from the bacterial culture and the phenotypic susceptibility assay was carried out and analyzed as described in Example 1. The nucleic acid (DNA) sequence of the patient derived HIV-1 protease and reverse transcriptase regions was determined using a fluorescence detection chain termination cycle sequencing methodology (ABI/PE). The method was used to determine a consensus nucleic acid sequence representing the combination of sequences of the mixture of HIV-1 variants existing in the subject sample (representing the quasispecies), and to determine the nucleic acid sequences of individual variants. Genotypes are analyzed as lists of

amino acid differences between virus in the patient sample and a reference laboratory strain of HIV-1, NL4-3. Genotypes and corresponding phenotypes (fold-change in IC50 values) are entered in a relational database linking

5      these two results with patient information. Large datasets can then be assembled from patient virus samples sharing particular characteristics, such as the presence of any given mutation or reduced susceptibility to any drug or combination of drugs.

10

**(a)   Protease   inhibitor   susceptibility   of   viruses containing mutations at amino acid 90 of HIV-1 Protease.**

Phenotypic susceptibility profiles of 333 patient virus

15     samples which contained a mutation at position 90 (L90M) but not at positions 30 or 50, which are primary mutations associated with resistance to nelfinavir and amprenavir, respectively) were analyzed. According to most published guidelines, such viruses are expected to be resistant to

20     ritonavir,     nelfinavir,     indinavir,     and     saquinavir. However, only 79.3% and 84.7% of these samples displayed reduced   susceptibility   to   saquinavir   and   indinavir, respectively, using a 2.5-fold threshold (Table 13), while 43.5%   and   53.8%   displayed   reduced   susceptibility   to

25     saquinavir and indinavir, respectively, using a 10-fold threshold (Table 14).   Thus, there was poor correlation between the presence of mutations at position 90 and saquinavir or indinavir susceptibility.

30

**(b) Indinavir susceptibility (fold change threshold 2.5) of viruses containing combinations of mutations at amino acid 90 and one secondary mutation in HIV-1 Protease.**

To explore the possibility that indinavir resistance in viruses containing a mutation at position 90 requires the presence of other specific mutations, decreased indinavir susceptibility (fold-change in IC50 greater than 2.5) in viruses containing L90M was correlated with the presence of mutations at other positions. This analysis revealed several other positions (most strongly 53, 95, 54, 84, 82 and 46) that decreased indinavir susceptibility significantly in combination with the L90M mutation, compared to when these other mutations were absent (see Table 18). The presence of a mutation at position 13 or 74 significantly decreased the proportion of samples that had reduced indinavir susceptibility (Table 18). In other words, the absence of mutations at position 13 or 74 was correlated with decreased susceptibility to indinavir.

**(c) Indinavir susceptibility (fold change threshold 10) of viruses containing combinations of mutations at amino acid 90 and one secondary mutation in HIV-1 Protease.**

To explore the possibility that indinavir resistance in viruses containing a mutation at position 90 requires the presence of other specific mutations, decreased indinavir susceptibility (fold-change in IC50 greater than 10) in viruses containing L90M was correlated with the presence of mutations at other positions. This analysis revealed several secondary positions (most strongly 95, 55, 54, 82, 85, 84, 20, 72, and 62) that decreased indinavir susceptibility significantly in combination with the L90M

mutation, compared to when these other mutations were absent (see Table 19). The presence of a mutation at position 74 significantly decreased the proportion of samples that had reduced indinavir susceptibility (27.5% vs. 57.3%; Table 19). In other words, the absence of a mutation at position 74 was correlated with decreased susceptibility to indinavir.

**(d) Saquinavir susceptibility (fold change threshold 2.5) of viruses containing combinations of mutations at amino acid 90 and one secondary mutation in HIV-1 Protease.**

To explore the possibility that saquinavir resistance in viruses containing a mutation at position 90 requires the presence of other specific mutations, decreased saquinavir susceptibility (fold-change in IC50 greater than 2.5) in viruses containing L90M was correlated with the presence of mutations at other positions. This analysis revealed several other positions (most strongly 53, 66, 84, 54, 48, 33, 73, 20, and 71) that decreased saquinavir susceptibility significantly in combination with the L90M mutation, compared to when these other mutations were absent (see Table 20). The presence of a mutation at position 64 or 93 significantly decreased the proportion of samples that had reduced saquinavir susceptibility (Table 20). In other words, the absence of a mutation at position 64 or 93 was correlated with decreased susceptibility to saquinavir.

**(e) Saquinavir susceptibility (fold change threshold 10) of viruses containing combinations of mutations at amino acid 90 and one secondary mutation in HIV-1 Protease.**

To explore the possibility that saquinavir resistance in viruses containing a mutation at position 90 requires the presence of other specific mutations, decreased saquinavir susceptibility (fold-change in IC50 greater than 10) in viruses containing L90M was correlated with the presence of mutations at other positions. This analysis revealed several other positions (most strongly 84, 53, 48, 23, 58, 20, 36, and 54) that decreased saquinavir susceptibility significantly in combination with the L90M mutation, compared to when these other mutations were absent (see Table 21). The presence of a mutation at position 64, 77 or 93 significantly decreased the proportion of samples that had reduced saquinavir susceptibility (Table 21). In other words, the absence of a mutation at position 64, 77 or 93 was correlated with decreased susceptibility to saquinavir.

**(f) Saquinavir susceptibility (fold change threshold 2.5) of viruses containing combinations of mutations at amino acid 90 and many secondary mutations in HIV-1 Protease.**

To explore the possibility that saquinavir resistance in viruses containing mutations at position 90 requires the presence of some defined number of other mutations, decreased saquinavir susceptibility (fold-change in IC50 greater than 2.5) in viruses containing L90M was correlated with the number of mutations at secondary positions. The following positions were considered: 10, 20, 52, 53, 54, 66, 71, 73, and 84; positions 53 and 84

were weighted twice, yielding a saquinavir resistance-associated mutation count. This analysis revealed that 100% of samples with L90M and a mutation count of at least 5 had reduced saquinavir susceptibility (See Table 22). Combination with 3 or 4 other secondary mutations also significantly increased the proportion of samples that had reduced saquinavir susceptibility (85.7% and 97.3%, respectively; see Table 22).

**Table 13. PRI Susceptibility of Viruses with Mutations at 82 and/or 90 (fold change threshold > 2.5).**

| Drug | Percent of viruses with indicated primary mutation(s) with reduced susceptibility (fold change in $IC_{50}$ > 2.5) | |
|---|---|---|
| | V82 mutations | L90M |
| Amprenavir | 60.0 | 60.4 |
| Indinavir | 92.2 | 84.7 |
| Nelfinavir | 94.4 | 97.0 |
| Ritonavir | 97.8 | 93.4 |
| Saquinavir | 61.7 | 79.3 |

**Table 14. PRI Susceptibility of Viruses with Mutations at 82 and/or 90 (fold change threshold > 10).**

| Drug | Percent of viruses with indicated primary mutation(s) with reduced susceptibility (fold change in $IC_{50}$ > 10) | |
|---|---|---|
| | V82 mutations | L90M |
| Amprenavir | 10.4 | 12.9 |
| Indinavir | 60.0 | 53.8 |
| Nelfinavir | 68.9 | 74.5 |
| Ritonavir | 89.3 | 66.1 |
| Saquinavir | 31.2 | 43.5 |

Table 15. Correlation Between V82 mutations, Secondary Mutations, and Indinavir Susceptibility (fold change threshold > 10).

| position | + or – | n | mt % >10 | wt %>10 | p value |
|---|---|---|---|---|---|
| 73 | + | 22 | 90.9 | 57.3 | 0.0011 |
| 55 | + | 25 | 80.0 | 58.0 | 0.0238 |
| 48 | + | 35 | 77.1 | 57.4 | 0.0188 |
| 20 | + | 77 | 76.6 | 53.4 | <0.001 |
| 43 | + | 34 | 76.5 | 57.6 | 0.0258 |
| 53 | + | 33 | 75.8 | 57.8 | 0.0349 |
| 90 | + | 135 | 74.1 | 45.9 | <0.001 |
| 72 | + | 56 | 73.2 | 56.5 | 0.0160 |
| 35 | + | 91 | 72.5 | 53.6 | 0.0019 |
| 54 | + | 188 | 71.3 | 34.1 | <0.001 |
| 71 | + | 184 | 70.7 | 37.2 | <0.001 |
| 36 | + | 99 | 69.7 | 54.4 | 0.0091 |
| 10 | + | 224 | 66.1 | 30.4 | <0.001 |
| 82 | | 270 | 60.0 | | |
| 13 | – | 37 | 45.9 | 62.2 | 0.0458 |

Table 16. Correlation Between V82 mutations, Secondary Mutations, and Saquinavir Susceptibility (fold change threshold > 2.5)

| position | + or – | n | mt % >2.5 | wt %>2.5 | p value |
|---|---|---|---|---|---|
| 84 | + | 36 | 100.0 | 55.8 | <0.001 |
| 48 | + | 35 | 97.1 | 56.4 | <0.001 |
| 23 | + | 11 | 90.9 | 60.5 | 0.0358 |
| 73 | + | 22 | 90.9 | 59.1 | 0.0018 |
| 53 | + | 33 | 87.9 | 58.1 | <0.001 |
| 33 | + | 24 | 87.5 | 59.2 | 0.0041 |
| 74 | + | 25 | 84.0 | 59.4 | 0.0113 |
| 20 | + | 77 | 83.1 | 53.1 | <0.001 |
| 90 | + | 135 | 82.2 | 41.0 | <0.001 |
| 43 | + | 34 | 79.4 | 59.1 | 0.0162 |
| 36 | + . | 99 | 75.8 | 53.5 | <0.001 |
| 41 | + | 79 | 74.7 | 56.3 | 0.0032 |
| 54 | + | 187 | 74.3 | 32.9 | <0.001 |
| 71 | + | 183 | 74.3 | 34.9 | <0.001 |
| 35 | + | 91 | 73.6 | 55.6 | 0.0028 |
| 10 | + | 223 | 69.5 | 23.9 | <0.001 |
| 82 | | 269 | 61.7 | | |
| 32 | – | 24 | 37.5 | 64.1 | 0.0106 |
| 39 | – | 4 | 0.0 | 62.6 | 0.0207 |

Table 17. Correlation Between V82 mutations, Secondary Mutations, and Saquinavir Susceptibility (fold change threshold > 10)

| position | + or − | n | mt % >10 | wt %>10 | p value |
|---|---|---|---|---|---|
| 48 | + | 35 | 82.9 | 23.5 | <0.001 |
| 23 | + | 11 | 81.8 | 29.1 | <0.001 |
| 84 | + | 36 | 72.2 | 24.9 | <0.001 |
| 53 | + | 33 | 69.7 | 25.8 | <0.001 |
| 74 | + | 25 | 56.0 | 28.7 | 0.0062 |
| 20 | + | 77 | 55.8 | 21.4 | <0.001 |
| 60 | + | 30 | 50.0 | 28.9 | 0.0181 |
| 33 | + | 24 | 50.0 | 29.4 | 0.0352 |
| 36 | + | 99 | 47.5 | 21.8 | <0.001 |
| 35 | + | 91 | 44.0 | 24.7 | 0.0011 |
| 90 | + | 135 | 43.0 | 19.4 | <0.001 |
| 41 | + | 79 | 41.8 | 26.8 | 0.0126 |
| 62 | + | 119 | 41.2 | 23.3 | 0.0013 |
| 54 | + | 187 | 39.0 | 13.4 | <0.001 |
| 71 | + | 183 | 37.7 | 17.4 | <0.001 |
| 10 | + | 223 | 35.0 | 13.0 | 0.0019 |
| 82 | | 269 | 31.2 | | |
| 46 | − | 156 | 26.3 | 38.1 | 0.0275 |
| 32 | − | 24 | 12.5 | 33.1 | 0.0268 |

Table 18. Correlation Between L90M, Secondary Mutations, and Indinavir Susceptibility (fold change threshold > 2.5).

| position | + or − | n | mt % >2.5 | wt %>2.5 | p value |
|---|---|---|---|---|---|
| 53 | + | 29 | 100.0 | 83.2 | 0.0064 |
| 95 | + | 23 | 100.0 | 83.5 | 0.0189 |
| 54 | + | 129 | 98.4 | 76.0 | <0.001 |
| 84 | + | 104 | 97.1 | 79.0 | <0.001 |
| 82 | + | 135 | 94.1 | 78.3 | <0.001 |
| 46 | + | 164 | 93.3 | 76.3 | <0.001 |
| 73 | + | 117 | 92.3 | 80.6 | 0.0027 |
| 71 | + | 233 | 91.4 | 69.0 | <0.001 |
| 20 | + | 115 | 91.3 | 81.2 | 0.0095 |
| 10 | + | 255 | 90.2 | 66.7 | <0.001 |
| 63 | + | 325 | 85.5 | 50.0 | 0.0214 |
| 90 | | 333 | 84.7 | | |
| 13 | − | 77 | 76.6 | 87.1 | 0.0226 |
| 74 | − | 40 | 67.5 | 87.0 | 0.0028 |

Table 19. Correlation Between L90M, Secondary Mutations, and Indinavir Susceptibility (fold change threshold > 10).

| position | + or - | n | mt % >10 | wt %>10 | p value |
|---|---|---|---|---|---|
| 95 | + | 23 | 82.6 | 51.6 | 0.0030 |
| 55 | + | 22 | 81.8 | 51.8 | 0.0048 |
| 54 | + | 129 | 81.4 | 36.3 | <0.001 |
| 82 | + | 135 | 74.1 | 39.9 | <0.001 |
| 85 | + | 23 | 73.9 | 52.3 | 0.0346 |
| 84 | + | 104 | 70.2 | 46.3 | <0.001 |
| 20 | + | 115 | 66.1 | 47.2 | 0.0103 |
| 72 | + | 87 | 64.4 | 50.0 | 0.0141 |
| 62 | + | 154 | 63.6 | 45.3 | <0.001 |
| 46 | + | 164 | 63.4 | 44.4 | <0.001 |
| 36 | + | 114 | 63.2 | 48.9 | 0.0088 |
| 10 | + | 255 | 63.1 | 23.1 | <0.001 |
| 71 | + | 233 | 60.9 | 37.0 | <0.001 |
| 90 | | 333 | 53.8 | | |
| 74 | - | 40 | 27.5 | 57.3 | <0.001 |

Table 20. Correlation Between L90M, Secondary Mutations, and Saquinavir Susceptibility (fold change threshold > 2.5).

| position | + or - | n | mt % >2.5 | wt %>2.5 | p value |
|---|---|---|---|---|---|
| 53 | + | 29 | 100.0 | 77.3 | <0.001 |
| 66 | + | 13 | 100.0 | 78.4 | 0.0459 |
| 84 | + | 104 | 98.1 | 70.7 | <0.001 |
| 54 | + | 129 | 96.9 | 68.1 | <0.001 |
| 48 | + | 22 | 95.5 | 78.1 | 0.0362 |
| 33 | + | 37 | 94.6 | 77.4 | 0.0076 |
| 73 | + | 117 | 89.7 | 73.6 | <0.001 |
| 20 | + | 115 | 89.6 | 73.9 | <0.001 |
| 71 | + | 233 | 88.4 | 58.0 | <0.001 |
| 36 | + | 114 | 87.7 | 74.9 | 0.0038 |
| 10 | + | 255 | 86.3 | 56.4 | <0.001 |
| 37 | + | 104 | 85.6 | 76.4 | 0.0365 |
| 63 | + | 325 | 80.3 | 37.5 | 0.0112 |
| 90 | | 333 | 79.3 | | |
| 93 | - | 187 | 74.3 | 85.6 | 0.0080 |
| 64 | - | 66 | 63.6 | 83.1 | <0.001 |

Table 21. Correlation Between L90M, Secondary Mutations, and Saquinavir Susceptibility (fold change threshold > 10).

| position | + or - | n | mt % >10 | wt %>10 | p value |
|----------|--------|-----|----------|---------|---------|
| 84 | + | 104 | 84.6 | 24.9 | <0.001 |
| 53 | + | 29 | 82.8 | 39.8 | <0.001 |
| 48 | + | 22 | 81.8 | 40.8 | <0.001 |
| 23 | + | 12 | 75.0 | 42.4 | 0.0260 |
| 58 | + | 30 | 63.3 | 41.6 | 0.0182 |
| 20 | + | 115 | 61.7 | 33.9 | <0.001 |
| 36 | + | 114 | 61.4 | 34.2 | <0.001 |
| 54 | + | 129 | 60.5 | 32.8 | <0.001 |
| 35 | + | 109 | 53.2 | 38.8 | 0.0091 |
| 73 | + | 117 | 51.3 | 39.4 | 0.0240 |
| 10 | + | 255 | 50.6 | 20.5 | <0.001 |
| 71 | + | 233 | 49.8 | 29.0 | <0.001 |
| 62 | + | 154 | 49.4 | 38.5 | 0.0306 |
| 90 | | 333 | 43.5 | | |
| 93 | − | 187 | 38.0 | 50.7 | 0.0135 |
| 77 | − | 139 | 35.3 | 49.5 | 0.0066 |
| 64 | − | 66 | 33.3 | 46.1 | 0.0409 |

Table 22. Association Between Saquinavir Susceptibility, L90M, and Number of Resistance Associated Mutations.

| Number of secondary mutations | n | % with SQV FC > 2.5 | Mean SQV fold change |
|-------------------------------|-----|---------------------|----------------------|
| 0 | 17 | 23.5 | 2.4 |
| 1 | 40 | 25.0 | 2.4 |
| 2 | 49 | 69.4 | 5.4 |
| 3 | 63 | 85.7 | 10.0 |
| 4 | 74 | 97.3 | 36.6 − |
| 5 | 34 | 100 | 50.3 |
| 6 or more | 56 | 100 | 94.2 |

-187-

Tables 23-27 show results as indicated using the above procedures as described in Examples 17 and 18.

**Table 23. Correlation Between L90M, Secondary Mutations, and Amprenavir Susceptibility (fold change threshold > 2.5).**

Amprenavir

| position | + or - | n | mt % >2.5 | wt %>2.5 | p value |
|---|---|---|---|---|---|
| 89 | + | 11 | 90.9 | 59.3 | 0.0298 |
| 53 | + | 29 | 89.7 | 57.6 | <0.001 |
| 84 | + | 104 | 86.5 | 48.5 | <0.001 |
| 33 | + | 37 | 83.8 | 57.4 | 0.0012 |
| 92 | + | 24 | 83.3 | 58.6 | 0.0120 |
| 95 | + | 23 | 82.6 | 58.7 | 0.0174 |
| 54 | + | 129 | 80.6 | 47.5 | <0.001 |
| 58 | + | 30 | 76.7 | 58.7 | 0.0400 |
| 46 | + | 164 | 75.0 | 46.2 | <0.001 |
| 82 | + | 135 | 70.4 | 53.5 | 0.0014 |
| 36 | + | 114 | 70.2 | 55.3 | 0.0055 |
| 10 | + | 255 | 69.4 | 30.8 | <0.001 |
| 62 | + | 154 | 66.2 | 55.3 | 0.0272 |
| 90 | | 333 | 60.4 | | |
| 74 | - | 40 | 45.0 | 62.5 | 0.0269 |
| 15 | - | 53 | 43.4 | 63.6 | 0.0050 |

**Table 24. Correlation Between L90M, Secondary Mutations, and Amprenavir Susceptibility (fold change threshold > 10).**

Amprenavir

| position | + or - | n | % >10 | wt %>10 | p value |
|---|---|---|---|---|---|
| 47 | + | 5 | 80.0 | 11.9 | 0.0011 |
| 33 | + | 37 | 48.6 | 8.4 | <0.001 |
| 66 | + | 13 | 38.5 | 11.9 | 0.0166 |
| 32 | + | 16 | 37.5 | 11.7 | 0.0097 |
| 55 | + | 22 | 31.8 | 11.6 | 0.0140 |
| 53 | + | 29 | 27.6 | 11.5 | 0.0213 |
| 54 | + | 129 | 24.0 | 5.9 | <0.001 |
| 84 | + | 104 | 22.1 | 8.7 | 0.0010 |
| 13 | + | 77 | 19.5 | 10.9 | 0.0424 |
| 46 | + | 164 | 17.7 | 8.3 | 0.0080 |
| 10 | + | 255 | 16.1 | 2.6 | <0.001 |
| 90 | | 333 | 12.9 | | |
| 69 | - | 37 | 2.7 | 14.2 | 0.0316 |

-188-

### Table 25. Correlation Between V82, Secondary Mutations, and Indinavir Susceptibility (fold change threshold > 2.5)

Indinavir

| position | + or − | n | mt % >2.5 | wt %>2.5 | p value |
|---|---|---|---|---|---|
| 84 | + | 37 | 100.0 | 91.0 | 0.0397 |
| 20 | + | 77 | 98.7 | 89.6 | 0.0064 |
| 72 | + | 56 | 98.2 | 90.7 | 0.0432 |
| 54 | + | 188 | 97.3 | 80.5 | <0.001 |
| 71 | + | 184 | 97.3 | 81.4 | <0.001 |
| 46 | + | 157 | 95.5 | 87.6 | 0.0155 |
| 93 | + | 133 | 95.5 | 89.1 | 0.0391 |
| 10 | + | 224 | 94.6 | 80.4 | 0.0034 |
| 82 | | 270 | 92.2 | | |
| 37 | − | 108 | 88.0 | 95.1 | 0.0297 |
| 64 | − | 56 | 85.7 | 93.9 | 0.0451 |
| 13 | − | 37 | 70.3 | 95.7 | <0.001 |
| 45 | − | 12 | 58.3 | 93.8 | <0.001 |

### Table 26. Correlation Between V82, Secondary Mutations, and Amprenavir Susceptibility (fold change threshold > 2.5)

Amprenavir

| position | + or − | n | mt % >2.5 | wt %>2.5 | p value |
|---|---|---|---|---|---|
| 33 | + | 24 | 95.8 | 56.5 | <0.001 |
| 23 | + | 12 | 91.7 | 58.5 | 0.0176 |
| 84 | + | 37 | 86.5 | 55.8 | <0.001 |
| 32 | + | 24 | 83.3 | 57.7 | 0.0104 |
| 53 | + | 33 | 81.8 | 57.0 | 0.0042 |
| 90 | + | 135 | 70.4 | 49.6 | <0.001 |
| 37 | + | 108 | 66.7 | 55.6 | 0.0442 |
| 71 | + | 184 | 66.3 | 46.5 | 0.0016 |
| 46 | + | 157 | 65.6 | 52.2 | 0.0184 |
| 10 | + | 224 | 65.2 | 34.8 | <0.001 |
| 54 | + | 188 | 63.8 | 51.2 | 0.0356 |
| 82 | | 270 | 60.0 | | |
| 61 | − | 21 | 38.1 | 61.8 | 0.0297 |

-189-

Table 27. Correlation Between V82, Secondary Mutations,
and Amprenavir Susceptibility (fold change threshold > 10).

Amprenavir

| position+ or − | | n | mt % >10 | wt %>10 | p value |
|---|---|---|---|---|---|
| 33 | + | 24 | 50.0 | 6.5 | <0.001 |
| 11 | + | 8 | 37.5 | 9.5 | 0.0394 |
| 84 | + | 37 | 35.1 | 6.4 | <0.001 |
| 32 | + | 24 | 25.0 | 8.9 | 0.0258 |
| 60 | + | 30 | 23.3 | 8.8 | 0.0229 |
| 53 | + | 33 | 21.2 | 8.9 | 0.0382 |
| 90 | + | 135 | 14.8 | 5.9 | 0.0133 |
| 46 | + | 157 | 14.6 | 4.4 | 0.0046 |
| 71 | + | 184 | 13.0 | 4.7 | 0.0243 |
| 10 | + | 224 | 12.5 | 0.0 | 0.0039 |
| 82 | | 270 | 10.4 | | |

In Tables 13-27, the first column lists the various codon
positions for HIV-1 protease for the secondary mutations
and the primary mutation at codon 82 or 90.

The second column represents a positive (+) or negative(-)
correlation between the change in resistance from the
number of wild-type reference samples to those samples
having the secondary mutation.

The fourth column, designates "mt %", as the percentage of
samples having the secondary mutation and showing the
indicated fold resistance to the specified protease
inhibitor, (i.e, > 10 fold or > 2.5 fold).

The fifth column, designates "wt %", as the percentage of
wild-type reference samples showing the indicated fold
resistance, (i.e, > 10 fold or or 2.5 fold) to the
specified protease inhibitor.

The sixth column represents the statistical P value for a
correlation.

The following list of mutations represents, by example, secondary mutations from a database for selected patient samples used to establish the above data in Tables 13-27. The mutations listed show the wild type reference amino acid and the possible various mutations for the substituted amino acid at the designated codon position for HIV-1 protease.

L10F/I/R/V, I13V, K20I/M/R/T/V
L23I, V32I, L33F/I/V
E35/D/N/G, M36I/L/T/V, N37C/D/E/G/H/S/T
P39A/Q/S/T, R41K/W/S, K43R/T
K45R, M46I/L/V, G48M/S/V
S53L/Y, I54A/L/M/S/T/V, K55N/R
Q58E, D60E, I62/V/M,
L63A/C/D/S/H/I/N/P/Q/R/S/T/V/Y
I64L/M/V, I66F/L/T/V, A71I/L/T/V
I72A/E/K/L/M/R/T/V, G73A/C/S/T, T74A/K/P/S
V77I/T, V82A/F/S/T, I84A/M/V, I85V
L90M, I93L/M, C95F

**Detailed Description of the Invention** (continued)

In another embodiment, the present invention provides a method for determining whether an HIV virus obtained from a patient infected with HIV is resistant to IDV, LPV, NFV and RTV which comprises determining whether a mutation at position 30 from D to N exists in the HIV protease obtained from the patient, wherein the presence o the mutation indicates that the virus is resistant to IDV, LPV, NFV and RTV.

In another embodiment, the present invention provides a method for determining whether an HIV virus obtained from a patient infected with HIV is resistant to IDV, LPV, NFV or RTV which comprises determining whether the virus is resistant to any one of IDV, LPV, NFV or RTV, wherein a determination that the virus is resistant to any one of IDV, LPV, NFV or RTV is indicative of the virus being resistant to IDV, LPV, NFV and RTV.

In a further embodiment, the present invention provides a method for determining cross resistance of an HIV virus to RTV and SQV which comprises determining (i) whether position 30 of the HIV protease is D, and (ii) whether the virus is resistant to NFV, wherein a mutation from D to N at position 30 of HIV protease and resistance of the virus to NFV are indicative of cross resistance to IDV and SQV.

In yet another embodi the invention provides a method for determining whether an HIV virus obtained from a patient infected with HIV is resistant to LPV and IND which comprises determining whether position 50 of the HIV protease of the virs is I or V, wherein the determination that position 50 is V is indicative of the virus being resistant to LPV and IND. These and other embodiments of the invention are shown and described with reference to the accompanying Figures 6-28 in view of the specification contained herein.

-192-

What is claimed is:

1.  A method of assessing the effectiveness of protease antiretroviral therapy of an HIV-infected patient comprising:

> (a)   collecting a plasma sample from the HIV-infected patient;
>
> (b)   evaluating whether the plasma sample contains nucleic acid encoding HIV protease having a mutation at codon 88; and
>
> (c)   determining increased susceptibility to amprenavir.

2.  The method of claim 1, wherein the mutation at codon 88 codes for a serine (S).

3.  The method of claim 1, wherein the HIV-infected patient is being treated with an antiretroviral agent.

4.  A method of assessing the effectiveness of protease antiretroviral therapy of an HIV-infected patient comprising:

> (a)   collecting a plasma sample from the HIV-infected patient;
>
> (b)   evaluating whether the plasma sample contains nucleic acid encoding HIV protease having a mutation at codon 88 and additional mutations at codons 63 and/or 77 or a combination thereof; and
>
> (c)   determining decreased susceptibilty to nelfinavir and indinavir and increased susceptibility to amprenavir.

5.  The method of claim 4, wherein the mutation at codon 63 codes for a proline (P) or a glutamine (Q) and the mutation at codon 77 codes for an isoleucine (I).

●

●

6.    The method of claim 4, wherein the HIV-infected patient is being treated with an antiretroviral agent.

7.    A method of assessing the effectiveness of protease antiretroviral therapy of an HIV-infected patient comprising:

(a)    collecting a plasma sample from the HIV-infected patient;

(b)    evaluating whether the plasma sample contains nucleic acid encoding HIV protease having a mutation at codon 88 and additional mutations at codons 63, 77 and/or 46 or a combination thereof; and

(c)    determining decreased susceptibilty to nelfinavir and indinavir and increased susceptibility to amprenavir.

8.    The method of claim 7, wherein the mutation at codon 63 codes for a proline (P) or a glutamine (Q), the mutation at codon 77 codes for an isoleucine (I) and the mutation at codon 46 codes for a leucine (L) or an isoleucine (I).

9.    The method of claim 7, wherein the HIV-infected patient is being treated with an antiretroviral agent.

10.    A method of assessing the effectiveness of protease antiretroviral therapy of an HIV-infected patient comprising:

(a)    collecting a plasma sample from the HIV-infected patient;

(b)    evaluating whether the plasma sample contains nucleic acid encoding HIV protease having a mutation at codon 88 and additional mutations at codons 63, 77, 46, 10, 20, and/or 36 or a

-194-

combination thereof; and

(c)    determining   decreased   susceptibilty   to
       nelfinavir   and   indinavir   and   increased
       susceptibility to amprenavir.

11.   The method of claim 10, wherein the mutation at codon
      63 codes for a proline (P) or a glutamine (Q), the
      mutation at codon 77 codes for an isoleucine (I), the
      mutation at codon 46 codes for a leucine (L) or an
      isoleucine (I), the mutation at codon 10 codes for a
      isoleucine (I) or a phenylalanine (F), the mutation at
      20 codes for a threonine (T) or a methionine (M) or an
      arginine (R), and the mutation at 36 codes for an
      isoleucine (I) or a valine (V).

12.   The method of claim 10, wherein the HIV-infected
      patient is being treated with an antiretroviral agent.

13.   A method for evaluating the biological effectiveness of
      a   candidate   HIV   antiretroviral   drug   compound
      comprising:

      (a)   introducing a resistance test vector comprising a
            patient-derived   segment   further   comprising   a
            mutation at codon 88 and an indicator gene into a
            host cell;

      (b)   culturing the host cell from step (a);    -

      (c)   measuring the indicator in a target host cell; and

      (d)   comparing the measurement of the indicator from
            step (c) with the measurement of the indicator
            measured when steps (a) - (c) are carried out in
            the absence of the candidate antiretroviral drug
            compound;

      wherein   a   test   concentration   of   the   candidate
      antiretroviral drug compound is present at steps (a) -
      (c); at steps (b) - (c); or at step (c).

14.　A method for evaluating the biological effectiveness of
a candidate HIV antiretroviral drug compound
comprising:

(a)　introducing a resistance test vector comprising a
patient-derived segment further comprising a
mutation at codon 88 and mutation(s) at codons 63
and/or 77 or a combination thereof and an
indicator gene into a host cell;

(b)　culturing the host cell from step (a);

(c)　measuring the indicator in a target host cell; and

(d)　comparing the measurement of the indicator from
step (c) with the measurement of the indicator
measured when steps (a) - (c) are carried out in
the absence of the candidate antiretroviral drug
compound;

wherein a test concentration of the candidate
antiretroviral drug copound is present at steps (a) -
(c); at steps (b) - (c); or at step (c).

15.　A method for evaluating the biological effectiveness of
a candidate HIV antiretroviral drug compound
comprising:

(a)　introducing a resistance test vector comprising a
patient-derived segment further comprising a
mutation at codon 88 and mutation(s) at codons 63,
77, and/or 46 or a combination thereof and an
indicator gene into a host cell;

(b)　culturing the host cell from step (a);

(c)　measuring the indicator in a target host cell; and

(d)　comparing the measurement of the indicator from
step (c) with the measurement of the indicator
measured when steps (a) - (c) are carried out in
the absence of the candidate antiretroviral drug
compound;

-196-

5      wherein a test concentration of the candidate antiretroviral
       drug compound is present at steps (a) - (c); at steps (b) -
       (c); or at step (c).


       16.  A method for evaluating the biological effectiveness of
10          a candidate HIV antiretroviral drug compound comprising:
            (a)  introducing a resistance test vector comprising a
                 patient-derived  segment  further  comprising  a
                 mutation at codon 88 and mutation(s) at codons 63,
                 77, 46, 10, 20, and/or 36 or a combination thereof
15               and an indicator gene into a host cell;
            (b)  culturing the host cell from step (a);
            (c)  measuring the indicator in a target host cell; and
            (d)  comparing the measurement of the indicator from
                 step (c) with the measurement of the indicator
20               measured when steps (a) - (c) are carried out in
                 the absence of the candidate antiretroviral drug
                 compound;
       wherein a test concentration of the candidate antiretroviral
       drug compound is present at steps (a) - (c); at steps (b) -
25     (c); or at step (c).


       17.  A resistance test vector comprising an HIV —
            patient-derived segment further comprising protease
            having a mutation at codon 88 and an indicator gene,
30          wherein  the  expression  of  the  indicator  gene  is
            dependent upon the patient derived segment.


       18.  The resistance test vector of claim 17, wherein the
            patient-derived segment having a mutation at codon 88
35          further comprises mutations at codons 63 and 77 or a
            combination thereof.


       19.  The resistance test vector of claim 17, wherein the
            patient-derived segment having a mutation at codon 88
40          further comprises mutations at codons 63, 77 and/or 46

5      or a combination thereof.

20. The resistance test vector of claim 17, wherein the
patient-derived segment having a mutation at codon 88
further comprises mutations at codons 63, 77, 46, 10,
10     20 and/or 36 or a combination thereof.

21. A method for evaluating the viral fitness of a
patient's virus comprising:
   (a) introducing a resistance test vector comprising a
15       patient-derived segment from a patient's virus and
         an indicator gene into a host cell;
   (b) culturing the host cell from step (a);
   (c) measuring the luciferase activity in a target host
       cell in the absence of any antiretroviral drug;
20       and
   (d) comparing the measurement of the indicator from
       step (c) with the measurement of the indicator
       measured when steps (a)-(c) are carried out for a
       reference control in the absence of any
25       antiretroviral drug;
wherein a reduction in the luciferase activity measured in
step (c) as compared to step (d) indicates a reduction in
viral fitness.

30     22. A method of assessing the effectiveness of protease
antiretroviral therapy of an HIV-infected patient comprising:
   (a) collecting a plasma sample from the HIV-infected
       patient;
   (b) evaluating whether the plasma sample contains
35       nucleic acid encoding HIV protease having a
         mutation at codon 82 and secondary positions; and
   (c) determining changes in susceptibility to
       ritonavir, nelfinavir, indinavir, saquinivir and
       amprenavir.

40

5      23.   The method of claim 22, wherein the mutation at codon
             82 codes for alanine (A), phenylalanine (F), serine
             (S), or threonine (T).

       24.   The method of claim 22, wherein the HIV-infected
10           patient is being treated with an antiretroviral agent.

       25.   A method of assessing the effectiveness of protease
             antiretroviral therapy of an HIV-infected patient
             comprising:
15                  (a)  collecting    a    plasma   sample   from   the
                         HIV-infected patient;
                    (b)  evaluating whether the plasma sample contains
                         nucleic acid encoding HIV protease having a
                         mutation   at   codon   82   and   an   additional
20                       mutation at codon 24; and
                    (c)  determining   decreased   susceptibilty   to
                         indinavir.

       26.   The method of claim 25, wherein the mutation at codon
25           24 codes for an isoleucine (I).

       27.   The method of claim 25, wherein the HIV-infected
             patient is being treated with an antiretroviral agent.

30     28.   A method of assessing the effectiveness of protease
             antiretroviral therapy of an HIV-infected patient
             comprising:
                    (a)  collecting a plasma sample from the HIV-infected
                         patient;
35                  (b)  evaluating whether the plasma sample contains
                         nucleic acid encoding HIV protease having a
                         mutation at codon 82 and an additional mutation at
                         codon 71; and
                    (c)  determining decreased susceptibilty to indinavir.

10

-199-

29.  The method of claim 28, wherein the mutation at codon
     71 codes for an amino acid selected from the group
     consisting of a threonine, (T) valine, (V) leucine (L)
     and isoleucine (I).

30.  The method of claim 28, wherein the HIV-infected
     patient is being treated with an antiretroviral
     agent.

31.  A method of assessing the effectiveness of protease
     antiretroviral therapy of an HIV-infected patient
     comprising:
     (a)  collecting a plasma sample from the HIV-infected
          patient;
     (b)  evaluating whether the plasma sample contains
          nucleic acid encoding HIV protease having a
          mutation at codon 82 and additional mutations at
          codons selected from the group consisting of codon
          54, 46, 10, 63, and a combination thereof; and
     (c)  determining decreased susceptibilty to indinavir.

32.  The method of claim 31, wherein the mutation at codon
     54 codes for an amino acid selected from the group
     consisting of a valine (V), alanine (A), leucine (L)
     and threonine (T), the mutation at codon 46 codes for
     an amino acid selected from the group consisting of a
     leucine (L), isoleucine (I) and valine (V), the
     mutation at codon 10 codes for an amino acid selected
     from the group consisting of an isoleucine (I), valine
     (V), phenylalanine (F), and arginine (R), and the
     mutation at codon 63 codes for an amino acid selected
     from the group consisting of proline (P), alanine (A),
     serine (S), threonine (T), glutamine(Q), , cysteine
     (C),and valine (V).

-200-

33. The method of claim 31, wherein the HIV-infected patient is being treated with an antiretroviral agent.

34. A method of assessing the effectiveness of protease antiretroviral therapy of an HIV-infected patient comprising:

(a) collecting a plasma sample from the HIV-infected patient;

(b) evaluating whether the plasma sample contains nucleic acid encoding HIV protease having a mutation at codon 82 and an additional mutation at codon 20; and

(c) determining decreased susceptibilty to saquinavir.

35. The method of claim 34, wherein the mutation at codon 20 codes for an amino acid selected from the group consisting of a methionine (M), threonine (T), isoleucine (I), and arginine (R).

36. The method of claim 34, wherein the HIV-infected patient is being treated with an antiretroviral agent.

37. A method of assessing the effectiveness of protease antiretroviral therapy of an HIV-infected patient comprising:

(a) collecting a plasma sample from the HIV-infected patient;

(b) evaluating whether the plasma sample contains nucleic acid encoding HIV protease having a mutation at codon 82 and an additional mutation at codon 36; and

(c) determining decreased susceptibilty to saquinavir.

-201-

38. The method of claim 37, wherein the mutation at codon 36 for an amino acid selected from the group consisting of a isoleucine (I), leucine (L), and valine (V).

39. The method of claim 37, wherein the HIV-infected patient is being treated with an antiretroviral agent.

40. A method of assessing the effectiveness of protease antiretroviral therapy of an HIV-infected patient comprising:

    (a) collecting a plasma sample from the HIV-infected patient;

    (b) evaluating whether the plasma sample contains nucleic acid encoding HIV protease having a mutation at codon 82 and additional mutations at codons 24, 71, 54, and/or 10 or a combination thereof; and

    (c) determining decreased susceptibilty to saquinavir.

41. The method of claim 40, wherein the mutation at codon 24 codes for an isoleucine (I), the mutation at codon 71 codes for an amino acid selected from the group consisting of a threonine (T), valine (V), leucine (L), and isoleucine (I), the mutation at codon 54 codes for an amino acid selected from the group consisting of valine (V), alanine (A), leucine (L), and threonine (T), and the mutation at codon 10 codes for an amino acid selected from the group consisting of an isoleucine (I), valine (V), phenylalanine (F), and arginine(R).

42. The method of claim 40, wherein the HIV-infected patient is being treated with an antiretroviral agent.

-202-

43. A method of assessing the effectiveness of protease antiretroviral therapy of an HIV-infected patient comprising:

(a) collecting a plasma sample from the HIV-infected patient;

(b) evaluating whether the plasma sample contains nucleic acid encoding HIV protease having a mutation at codon 82 and the number of additional mutations at secondary positions; and

(c) determining decreased susceptibilty to indinavir and saquinavir.

44. The method of claim 43, wherein the number of additional mutations at secondary positions is at least 3.

45. A method of assessing the effectiveness of protease antiretroviral therapy of an HIV-infected patient comprising:

(ss) collecting a plasma sample from the HIV-infected patient;

(b) evaluating whether the plasma sample contains nucleic acid encoding HIV protease having a mutation at codon 90 and secondary mutations; and

(c) determining changes in susceptibility to ritonavir, nelfinavir, indinavir, saquinivir and amprenavir.

46. The method of claim 45, wherein the mutation at codon 90 codes for a methionine.

47. The method of claim 45, wherein the HIV-infected patient is being treated with an antiretroviral agent.

-203-

48. A method of assessing the effectiveness of protease antiretroviral therapy of an HIV-infected patient comprising:

(d) collecting a plasma sample from the HIV-infected patient;

(b) evaluating whether the plasma sample contains nucleic acid encoding HIV protease having a mutation at codon 90 and an additional mutation at codon 73; and

(c) determining decreased susceptibilty to indinavir.

49. The method of claim 48, wherein the mutation at codon 73 codes for an amino acid selected from the group consisting of a serine (S), threonine (T), and cysteine (C).

50. The method of claim 48, wherein the HIV-infected patient is being treated with an antiretroviral agent.

51. A method of assessing the effectiveness of protease antiretroviral therapy of an HIV-infected patient comprising:

(d) collecting a plasma sample from the HIV-infected patient;

(b) evaluating whether the plasma sample contains nucleic acid encoding HIV protease having a mutation at codon 90 and an additional mutation at codon 71; and

(c) determining decreased susceptibilty to indinavir.

52. The method of claim 51, wherein the mutation at codon 71 codes for an amino acid selected from the group consisting of a threonine (T), valine (V), leucine (L), and isoleucine (I).

53. The method of claim 51, wherein the HIV-infected patient is being treated with an antiretroviral agent.

54. A method of assessing the effectiveness of protease antiretroviral therapy of an HIV-infected patient comprising:
   (d) collecting a plasma sample from the HIV-infected patient;
   (b) evaluating whether the plasma sample contains nucleic acid encoding HIV protease having a mutation at codon 90 and an additional mutation at codon 46,; and
   (c) determining decreased susceptibilty to indinavir.

55. The method of claim 54, wherein the mutation at codon 46 codes for an amino acid selected from the group consisting of a leucine (L), isoleucine (I) and valine (V).

56. The method of claim 54, wherein the HIV-infected patient is being treated with an antiretroviral agent.

57. A method of assessing the effectiveness of protease antiretroviral therapy of an HIV-infected patient comprising:
   (d) collecting a plasma sample from the HIV-infected patient;
   (b) evaluating whether the plasma sample contains nucleic acid encoding HIV protease having a mutation at codon 90 and an additional mutation at codon 73; and
   (c) determining decreased susceptibilty to saquinavir.

58. The method of claim 57, wherein the mutation at codon 73 codes for an amino acid selected from the group

-205-

5   consisting of a serine (S), threonine (T), and cysteine (C).

59.  The method of claim 57, wherein the HIV-infected patient is being treated with an antiretroviral
10   agent.

60.  A method of assessing the effectiveness of protease antiretroviral therapy of an HIV-infected patient comprising:
15   (d)  collecting a plasma sample from the HIV-infected patient;

(b)  evaluating whether the plasma sample contains nucleic acid encoding HIV protease having a mutation at codon 90 and an additional mutation at
20   codon 71; and

(c)  determining decreased susceptibilty to saquinavir.

61.  The method of claim 60, wherein the mutation at codon 71 codes for an amino acid selected from the group
25   consisting of a threonine (T), valine (V), leucine (L), and isoleucine (I).

62.  The method of claim 60, wherein the HIV-infected patient is being treated with an antiretroviral
30   agent.

63.  A method of assessing the effectiveness of protease antiretroviral therapy of an HIV-infected patient comprising:
35   (d)  collecting a plasma sample from the HIV-infected patient;

(b)  evaluating whether the plasma sample contains nucleic acid encoding HIV protease having a mutation at codon 90 and additional mutations at
40   codons 77 and 10; and

-206-

5    (c)    determining decreased susceptibilty to saquinavir.

64.    The method of claim 63, wherein the mutation at codon
       77 codes for an amino acid selected from the group
       consisting of isoleucine (I) and threonine (T) and the
10     mutation at codon 10 codes for an amino acid selected
       from the group consisting of isoleucine (I), valine
       (V), phenylalanine (F), and arginine (R).

65.    The method of claim 63, wherein the HIV-infected
15     patient  is  being  treated  with  an  antiretroviral
       agent.

66.    A method of assessing the effectiveness of protease
       antiretroviral  therapy  of  an  HIV-infected  patient
20     comprising:
       (d)    collecting a plasma sample from the HIV-infected
              patient;
       (b)    evaluating whether the plasma sample contains
              nucleic  acid  encoding  HIV  protease  having  a
25            mutation at codon 90 and the number of additional
              mutations at secondary positions; and
       (c)    determining decreased susceptibilty to indinavir
              and saquinavir.

30  67.   The method of claim 66, wherein the number of additional
          mutations at secondary positions is at least 3.

    68.    A method of assessing the effectiveness of protease
           antiretroviral  therapy  of  an  HIV-infected  patient
35         comprising:
           (a)    collecting a plasma sample from the HIV-infected
                  patient;
           (b)    evaluating whether the plasma sample contains
                  nucleic acid encoding HIV protease having a mutation at
40                codons 82 and 90 and secondary mutations; and

-207-

(c)    determining    changes    in    susceptibility    to
ritonavir,   nelfinavir,   indinavir,   saquinivir   and
amprenavir.

69.    The method of claim 68, wherein the mutation at codon
82 codes for an amino acid selected from the group
consisting of alanine (A), phenylalanine (F), serine
(S), and threonine (T) and the mutation at codon 90
codes for a methionine (M).

70.    The method of claim 68, wherein the HIV-infected
patient  is  being  treated  with  an  antiretroviral
agent.

71.    A method for evaluating the biological effectiveness of
a candidate HIV protease antiretroviral drug compound
comprising:
       (a)    introducing a resistance test vector comprising a
              patient-derived  segment  further  comprising  a
              mutation at codon 82 and additional mutations at
              one or more secondary positions and an indicator
              gene into a host cell;
       (b)    culturing the host cell from step (a);
       (c)    measuring the indicator in a target host cell; and

       (d)    comparing the measurement of the indicator from
              step (c) with the measurement of the indicator
              measured when steps (a) - (c) are carried out in
              the absence of the candidate antiretroviral drug
              compound;

       wherein   a   test   concentration   of   the   candidate
       antiretroviral drug compound is present at steps (a) -
       (c); at steps (b) - (c); or at step (c).

-208-

72. A method for evaluating the biological effectiveness of a candidate HIV protease antiretroviral drug compound comprising:

(a) introducing a resistance test vector comprising a patient-derived segment further comprising a mutation at codon 82 and secondary mutation(s) at codons 20, 24, 71, 54 and/or 10 or a combination thereof and an indicator gene into a host cell;

(b) culturing the host cell from step (a);

(c) measuring the indicator in a target host cell; and

(d) comparing the measurement of the indicator from step (c) with the measurement of the indicator measured when steps (a) - (c) are carried out in the absence of the candidate antiretroviral drug compound;

wherein a test concentration of the candidate antiretroviral drug compound is present at steps (a) - (c); at steps (b) - (c); or at step (c).

73. A method for evaluating the biological effectiveness of a candidate HIV protease antiretroviral drug compound comprising:

(a) introducing a resistance test vector comprising a patient-derived segment further comprising a mutation at codon 90 and additional mutations at one or more secondary positions and an indicator gene into a host cell;

(b) culturing the host cell from step (a);

(c) measuring the indicator in a target host cell; and

(d) comparing the measurement of the indicator from step (c) with the measurement of the indicator measured when steps (a) - (c) are carried out in the absence of the candidate antiretroviral drug compound;

●                    ●

-209-

wherein a test concentration of the candidate antiretroviral drug compound is present at steps (a) - (c); at steps (b) - (c); or at step (c).

74. A method for evaluating the biological effectiveness of a candidate HIV protease antiretroviral drug compound comprising:

(a) introducing a resistance test vector comprising a patient-derived segment further comprising a mutation at codon 90 and secondary mutation(s) at codons 73, 71, 10 and/or 46 or a combination thereof and an indicator gene into a host cell;

(b) culturing the host cell from step (a);

(c) measuring the indicator in a target host cell; and

(d) comparing the measurement of the indicator from step (c) with the measurement of the indicator measured when steps (a) - (c) are carried out in the absence of the candidate antiretroviral drug compound;

wherein a test concentration of the candidate antiretroviral drug compound is present at steps (a) - (c); at steps (b) - (c); or at step (c).

75. A method for evaluating the biological effectiveness of a candidate HIV protease antiretroviral drug compound comprising:

(a) introducing a resistance test vector comprising a patient-derived segment further comprising a mutation at codons 82 and 90 and additional mutations at one or more secondary positions and an indicator gene into a host cell;

(b) culturing the host cell from step (a);

(c) measuring the indicator in a target host cell; and

(d) comparing the measurement of the indicator from step (c) with the measurement of the indicator

-210-

5          measured when steps (a) - (c) are carried out in
           the absence of the candidate antiretroviral drug
           compound;

           wherein a test concentration of the candidate
10         antiretroviral drug compound is present at steps (a) -
           (c); at steps (b) - (c); or at step (c).

76.        A resistance test vector comprising an HIV patient-
           derived segment further comprising protease having a
15         mutation at codon 82 and an indicator gene, wherein the
           expression of the indicator gene is dependent upon the
           patient derived segment.

77.        The resistance test vector of claim 76, wherein the
20         patient-derived segment having a mutation at codon 82
           further comprises at least one secondary mutation at
           a codon selected from the group consisting of 20, 24,
           71, 54, 10 and a combination thereof.

25    78.  The resistance test vector of claim 76, wherein the
           patient-derived segment having a mutation at codon 90
           further comprises at least one secondary mutation at a
           codon selected from the group consisting of 73, 71, 46,
           10 and a combination thereof.

30

79.        A method for determining replication capacity for a
           patient's virus comprising:
           (a)  introducing a resistance test vector comprising a
                patient derived segment and an indicator gene into
35              a host cell;
           (b)  culturing the host cell from (a);
           (c)  harvesting viral particles from step (b) and
                infecting target host cells;
           (d)  measuring expression of the indicator gene in the
10              target host cell, wherein the expression of the

-211-

5    indicator gene is dependent upon the patient-
     derived segment;

(e)    comparing the expression of the indicator gene
       from (d) with the expression of the indicator gene
       measured when steps (a) through (d) are carried
10     out in a control resistance test vector; and

(f)    normalizing the expression of the indicator gene
       by measuring an amount of virus in step (c).

80.    A method of assessing the effectiveness of protease
15     antiretroviral therapy of an HIV-infected patient
       comprising:

(a)    collecting a biological sample from the
       HIV-infected patient;

(b)    evaluating whether the biological sample contains
20     nucleic acid encoding HIV protease having a
       mutation at codon 82 or codon 90; and

(c)    determining changes in susceptibility to protease
       inhibitors.

25    81.    The method of claim 80, wherein step (c) determines
              changes in susceptibility to saquinavir.

      82..   The method of claim 80, wherein the mutation at codon
              82 codes for alanine (A), phenylalanine (F), serine
30            (S), or threonine (T).

      83.    The method of claim 82, wherein the mutation at codon
              82 is a substitution of alanine (A), phenylalanine (F),
              serine (S), or threonine (T) for valine(V).

35

      84.    The method of claim 80, wherein the mutation at codon
              90 codes for methionine (M).

      85.    The method of claim 84, wherein the mutation at codon
10            90 is a substitution of methionine (M) for leucine (L).

86.  A method for evaluating the biological effectiveness of a candidate HIV protease antiretroviral drug compound comprising:

(a)  introducing a resistance test vector comprising a patient-derived segment having nucleic acid encoding HIV protease with a mutation at codon 82 or codon 90 and an indicator gene into a host cell;

(b)  culturing the host cell from step (a);

(c)  measuring the indicator gene in a target host cell; and

(d)  comparing the measurement of the indicator gene from step (c) with the measurement of the indicator gene measured when steps (a) - (c) are carried out in the absence of the candidate antiretroviral drug compound;

wherein a test concentration of the candidate antiretroviral drug compound is present at steps (a) - (c); at steps (b) - (c); or at step (c).

87.  A resistance test vector comprising an HIV patient-derived segment, further comprising protease having a mutation at codon 82 or codon 90 and an indicator gene, wherein the expression of the indicator gene is dependent upon the patient-derived segment.

88.  The resistance test vector of claim 87, wherein the patient-derived segment having a mutation at codon 82 codes for alanine (A), phenylalanine (F), serine (S), or threonine (T).

89.  The resistance test vector of claim 88, wherein the patient-derived segment having a mutation at codon 82 is a substitution of alanine (A), phenylalanine (F), serine (S), or threonine (T) for valine (V).

-213-

90. The resistance test vector of claim 87, wherein the patient-derived segment having a mutation at codon 90 codes for methionine (M).

91. The resistance test vector of claim 90, wherein the patient-derived segment having a mutation at codon 90 is a substitution of methionine (M) for leucine (L).

92. A method for determining replication capacity for a patient's virus comprising:

(a) introducing a resistance test vector comprising a patient-derived segment and an indicator gene into a host cell;

(b) culturing the host cell from (a);

(c) harvesting viral particles from step (b) and infecting target host cells;

(d) measuring expression of the indicator gene in the target host cell, wherein the expression of the indicator gene is dependent upon the patient-derived segment; and

(e) comparing the expression of the indicator gene from (d) with the expression of the indicator gene measured when steps (a) through (d) are carried out in a control resistance test vector.

93. The method of claim 92 further comprising the step of:

(f) normalizing the expression of the indicator gene by measuring an amount of virus in step (c).

94. The method of claim 92 wherein the patient-derived segment comprises nucleic acid encoding HIV integrase having a mutation at codon 66.

-214-

95. The method of claim 92 wherein the patient-derived segment comprises nucleic acid encoding HIV integrase having a mutation at codon 154.

96. The method of claim 94 wherein the patient-derived segment comprises nucleic acid encoding HIV integrase having an additional mutation at codon 153.

97. The method of claim 94 wherein the patient-derived segment comprises nucleic acid encoding HIV integrase having an additional mutation at codon 154.

98. A method of assessing the effectiveness of protease antiretroviral therapy of an HIV-infected patient comprising:

(uuuu)    collecting a biological sample from the HIV-infected patient;

(b)    evaluating whether the biological sample contains nucleic acid encoding HIV protease having a mutation at codon 82 and a secondary mutation at codons selected from the group consisting of 73, 55, 48, 20, 43, 53, 90, 13, 84, 23, 33, 74, 32, 39, 60, 36, and 35, or a mutation at codon 90 and a secondary mutation at codons selected from the group consisting of 53, 95, 54, 84, 82, 46, 13, 74, 55, 85, 20, 72, 62, 66, 84, 48, 33, 73, 71, 64, 93, 23, 58, and 36; and

(c)    determining a change in susceptibility to a protease inhibitor.

99. The method of claim 98, wherein the mutation at codon 82 is a substitution of alanine (A), phenylalanine (F), serine (S), or threonine (T) for valine (V) and the mutation at codon 90 is a substitution of methionine (M) for leucine (L).

100. The method of claim 99, wherein the protease inhibitor is selected from the group consisting of indinavir, amprenavir, and saquinavir.

101. The method of claim 100, having a mutation at codon 82 and a secondary mutation at codons selected from the group consisting of 84, 48, 23, 73, 53, 33, 74, 20, 90, 32 and 39 or a mutation at codon 90 and a secondary mutation at codons selected from the group consisting of 53, 66, 84, 54, 48, 33, 73, 20, 71, 64 and 93, wherein the protease inhibitor is saquinavir.

102. The method of claim 101, having a mutation at codon 82 and a secondary mutation at codons selected from the group consisting of 84, 48, 23, 73, 53, 33, 74, 20, and 90, or a mutation at codon 90 and a secondary mutation at codons selected from the group consisting of 53, 66, 84, 54, 48, 33, 73, 20, and 71, wherein the change in susceptibility in step (c) is a decrease in susceptibility to saquinavir.

103. The method of claim 101, having a mutation at codon 82 and a secondary mutation at codons 32 or 39, or a mutation at codon 90 and a secondary mutation at codons 64 or 93, wherein the change in susceptibility in step (c) is an increase in susceptibility to saquinavir.

104. The method of claim 100, having a mutation at codon 90 and a secondary mutation at codons selected from the group consisting of 53, 95, 54, 84, 82, 46, 13, and 74, wherein the protease inhibitor is indinavir.

105. The method of claim 104, having a mutation at codon 90 and a secondary mutation at codons selected from the group consisting of 53, 95, 54, 84, 82, and 46, wherein the change in susceptibility in step (c) is a decrease

5      in susceptibility to indinavir.

106. The method of claim 104, having a mutation at codon 90
     and a secondary mutation at codons 13 or 74, wherein the
     change in susceptibility in step (c) is an increase in
10     susceptibility to indinavir.

107. The method of claim 100, having a mutation at codon 82
     and a secondary mutation at codons selected from the
     group consisting of 73, 55, 48, 20, 43, 53, 90, 13, 48,
15     23, 84, 53, 74, 60, 33, 36, 35, 32, and 46 or a mutation
     at codon 90 and a secondary mutation at codons selected
     from the group consisting of 95, 55, 54, 82, 85, 84, 20,
     72, 62, 74, 53, 48, 23, 58, 36, 64, 77, and 93.

20   108. The method of claim 107, wherein the protease inhibitor
     is selected from the group consisting of indinavir,
     amprenavir, and saquinavir.

109. The method of claim 108, wherein step (c) is determining
25     a change in susceptibility to the protease inhibitor
     greater than 10 fold.

110. The method of claim 108, having a mutation at codon 82
     and a secondary mutation at codons selected from the
30     group consisting of 48, 23, 84, 53, 74, 20, 60, 33, 36,
     35, or a mutation at codon 90 and a secondary mutation
     at codons selected from the group consisting of 84, 53,
     48, 23, 58, 20, 36, and 54, wherein the change in
     susceptibility in step (c) is a decrease in
35     susceptibility to saquinavir.

111. The method of claim 108, having a mutation at codon 82
     and a secondary mutation at codons 32 or 46, or a
     mutation at codon 90 and a secondary mutation at codons
40     64, 77, or 93, wherein the change in susceptibility in

-217-

step (c) is an increase in susceptibility to saquinavir.

112. The method of claim 108, having a mutation at codon 82 and a secondary mutation at codons selected from the group consisting of 73, 55, 48, 20, 43, 53, and 90, or a mutation at codon 90 and a secondary mutation at codons selected from the group consisting of 95, 55, 54, 82, 85, 84, 20, 72, and 62, wherein the change in susceptibility in step (c) is a decrease in susceptibility to indinavir.

113. The method of claim 108, having a mutation at codon 82 and a secondary mutation at codon 13, or a mutation at codon 90 and a secondary mutation at codon 74, wherein the change in susceptibility in step (c) is an increase in susceptibility to indinavir.

114. A method of assessing the effectiveness of protease antiretroviral therapy of an HIV-infected patient comprising:

(kkkkk)    collecting a biological sample from the HIV-infected patient;

(b)    evaluating whether the biological sample contains nucleic acid encoding HIV protease having a mutation at codon 90 and secondary mutations of at least three codons; and

(c)    determining a decrease in susceptibility to saqinavir.

115. The method of claim 114, wherein in the evaluating step (b), the nucleic acid encoding HIV protease has secondary mutations of at least five codons.

116. The method of claim 114, wherein the secondary mutation are selected from the group consisting of codons 10, 20, 52, 53, 54, 66, 71, 73 and 84.

-218-

117. A method of assessing the effectiveness of protease
antiretroviral therapy of an HIV-infected patient
comprising:

(nnnnn) collecting a biological sample from the
HIV-infected patient;

(b) evaluating whether the biological sample contains
nucleic acid encoding HIV protease having a
mutation at codon 82 and secondary mutations at
codons selected from the group consisting of 33,
23, 84, 32, 53, 90, 37, 71, 46, 10, 54, 61, 11,
and 46, or a mutation at codon 90 and secondary
mutations at codons selected from the group
consisting of 89, 53, 84, 33, 92, 95, 54, 58, 46,
82, 36, 10, 62, 74, 15, 47, 66, 32, 55, 53, 13,
and 69; and

(c) determining a change in susceptibility to
amprenavir.

118. The method of claim 117, wherein the mutation at codon
82 is a substitution of alanine (A), phenylalanine (F),
serine (S), or threonine (T) for valine(V) and the
mutation at codon 90 is a substitution of methionine (M)
for leucine (L).

119. The method of claim 118, having a mutation at codon 82
and secondary mutations at codons selected from the
group consisting of 33, 23, 84, 32, 53, 90, 37, 71, 46,
10, 54, 11, and 46, or a mutation at codon 90 and
secondary mutations at codons selected from the group
consisting of 89, 53, 84, 33, 92, 95, 54, 58, 46, 82,
36, 10, 62, 47, 66, 32, 55, 53, and 13; wherein the
change in susceptibility in step (c) is a decrease in
susceptibility to saquinavir.

120. The method of claim 118, having a mutation at codon 82
and a secondary mutation at codon 61, or a mutation at

-219-

5    codon 90 and secondary mutations at codons 74, 15, or
     69, wherein the change in susceptibility in step (c) is
     an increase in susceptibility to saquinavir.

121.  A resistance test vector comprising an HIV patient-
10    derived segment comprising nucleic acid encoding
      protease having a mutation at codon 82 and secondary
      mutations at codons selected from the group consisting
      of 73, 55, 48, 20, 43, 53, 90, 13, 84, 23, 33, 74, 32,
      39, 60, 36, and 35, or a mutation at codon 90 and
15    secondary mutations at codons selected from the group
      consisting of 53, 95, 54, 84, 82, 46, 13, 74, 55, 85,
      20, 72, 62, 66, 84, 48, 33, 73, 71, 64, 93, 23, 58, and
      36 and an indicator gene, wherein the expression of the
      indicator gene is dependent upon the patient-derived
20    segment.

122.  The resistance test vector of claim 121, wherein the
      mutation of the patient derived segment at codon 82 is
      a substitution of alanine (A), phenylalanine (F), serine
25    (S), or threonine (T) for valine(V) and the mutation at
      codon 90 is a substitution of methionine (M) for leucine
      (L).

30

123.  A method for determining whether an HIV virus obtained
      from a patient infected with HIV is resistant to IDV,
      LPV, NFV and RTV which comprises determining whether a
      mutation at position 30 from D to N exists in the HIV
35    protease obtained from the patient, wherein the presence
      o the mutation indicates that the virus is resistant to
      IDV, LPV, NFV and RTV.

40

●                    ●

124. A method for determining whether an HIV virus obtained
     from a patient infected with HIV is resistant to IDV,
     LPV, NFV or RTV which comprises determining whether the
     virus is resistant to any one of IDV, LPV, NFV or RTV,
     wherein a determination that the virus is resistant to
     any one of IDV, LPV, NFV or RTV is indicative of the
     virus being resistant to IDV, LPV, NFV and RTV.

125. A method for determining cross resistance of an HIV
     virus to RTV and SQV which comprises determining (i)
     whether position 30 of the HIV protease is D, and (ii)
     whether the virus is resistant to NFV, wherein a
     mutation from D to N at position 30 of HIV protease and
     resistance of the virus to NFV are indicative of cross
     resistance to IDV and SQV.

126. A method for determining whether an HIV virus obtained
     from a patient infected with HIV is resistant to LPV and
     IND which comprises determining whether position 50 of
     the HIV protease of the virs is I or V, wherein the
     determination that position 50 is V is indicative of the
     virus being resistant to LPV and IND.

TABLE A

# Summary of Replication Capacity (RC) and Enzyme Function Results

| | LOW RC (<25% of Ref.*) | MEDIUM RC (26-75% of Ref.) | HIGH RC (>75% of Ref.) |
|---|---|---|---|
| % of Total Tested | 41% (55) | 45% (59) | 14% (19) |
| PR Processing Defects (%p41>10%) | 71% (39) | 24% (14) | 10% (2) |
| Impaired RT Activity (<25% of reference) | 14% (7) | 2% (1) | 0% |
| >10 mutations in Protease | 62% (34) | 22% (13) | 5% (1) |
| >10X reduced susceptibility to NFV | 63% (35) | 32% (19) | 16% (3) |

*Reference virus: NL4-3